THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXIX JUNE. 1948 NO. 3

ON THE SYNERGISM OF SOME GRAM-POSITIVE COCCI AND VACCINIA VIRUS.

C. H. LACK.

From the Lister Institute, Elstree, Herts.

Received for publication March 18, 1948.

It has been the experience in this Institute that in the production of vaccine lymph, if the bacterial flora of the vaccinial eruption on the vaccinifer is reduced by appropriate antiseptics, then the yield both of vaccinial pulp and of virus is greatly reduced. This result was obtained both by the partial sterilization of the seed virus (lapine) with 1 per cent phenol, which does not significantly affect the potency of the virus, or by spraying the scarified and inoculated area of the vaccinifer with brilliant green. The vaccinifer used here in routine production of lymph is the sheep.

Preliminary experiments, which will be described, indicate that certain strains of staphylococci caused a considerable increase in tissue damage and in yield of virus when they were inoculated together with bacteria-free elementary body suspensions of virus. This led to a study of the factors involved in this apparent synergistic action, and to some observations on the similar action of streptococci.

PART I: PRELIMINARY EXPERIMENTS ON MIXED INFECTION OF BACTERIA AND VACCINIA.

Experiment I. To determine the effect of phenol on lymph production.

Eight sheep were washed, shaved and scarified over one flank in the usual way. Four were vaccinated with non-phenolized lapine in saline, i.e. with the heavily contaminated lymph from a rabbit, and gave a mean weight of pulp of 81 g. with a viable count of the order of one thousand million organisms per g. Four sheep, vaccinated with the same volume of lapine containing 0·7 per cent phenol, yielded a mean weight of 56 g. of pulp with a viable count of the order of five hundred thousand organisms per g.

The lymphs were ground and 1 g. diluted in ten-fold dilutions to $1/10^{-7}$. An equal volume of each dilution was applied to scarified squares on a rabbit's flank and the degree of vaccinial eruption recorded on the fifth day. The pulps obtained from the phenolized and non-phenolized lapines showed the same titre of virus by this method of titration, i.e. confluent reactions at $1/10^{-4}$ and a few

scattered pocks at $1/10^{-7}$. Thus the total yield of virus from the use of phenolized lapine was approximately two-thirds of that obtained from non-phenolized seed.

Experiments have shown that 0·7 per cent phenol has no significant action on the infectivity of the virus, so it seemed probable that the greater bacterial population in the pulp of the non-phenolized lapine in some way enhanced the multiplication of the virus. The predominant organism in sheep pulp is *Staphylococcus pyogenes*, so further experiments were carried out using pure suspensions of vaccinia and staphylococci.

Experiment II. To determine whether staphylococci increase the multiplication of vaccinia virus in the scarified rabbit skin.

The staphylococci were incubated overnight at 37° C. in meat digest broth pH 7·4, the cultures spun down, washed three times and resuspended in saline to match a Brown No. 2 opacity tube. Half of each suspension was killed by heat.

The elementary body suspension (E.B.S.) of vaccinia virus was obtained by reconstituting a dried preparation of E.B.S. which had been prepared and stored at 2° C. in sealed ampoules in 1938 by Dr. McClean of this Institute. One strain of staphylococcus was obtained from a human abscess and is known as "Humphrey." The other, strain 961, was isolated from sheep pulp.

Two adult rabbits weighing approximately 3 kg. were clipped over their flanks. Rectangles of equal dimensions were drawn on the skin of each flank, and the enclosed areas scarified by passing a metal comb lightly over the skin with the same number of strokes for each. Equal volumes of E.B.S. and living staphylococci were mixed and rubbed into the scarified area of one flank, and the same volume of the same E.B.S. and killed staphylococci into a similar area on the other flank. The rabbits were killed on the fifth day and the pulps collected. The pulps were weighed, ground up with 5 ml. of 40 per cent glycerol saline and the suspensions titrated in ten-fold dilutions on other rabbits.

TABLE I.—*The Influence of Staphylococci on the Proliferation of Vaccinia Virus.*

Strain.	Inoculum.	Weight of pulp.	Vaccinial titre of pulp.
Staph. humphrey	E.B.S. live staph.	3·5 g.	Confluent reaction at 1/5000
	,, dead staph.	1 g.	Confluent reaction at 1/500
Staph. 961	E.B.S. live staph	3·1 g.	Confluent at 1/10,000
	,, dead staph.	1·5 g.	Confluent at 1/10,000

The results are set out in Table I, and suggest that living staphylococcus "Humphrey" caused a thirty-fold increase in the yield of virus, while strain 961 had little effect.

The reports of Duran-Reynals (1928), McClean (1930), Hoffman (1931) and others on the spread of vaccinia virus by hyaluronidase suggested that this enzyme might enhance proliferation of the virus when hyaluronidase-producing

staphylococci were present. The mucin clot prevention test (M.C.P.) for hyaluronidase production by these staphylococci showed that strain " Humphrey " gave a titre of 1/3200 and strain 961 a titre of less than 1/200. The details of this test are described in Part II of this paper. It seemed of interest, therefore, to study the factors responsible for this apparent synergism between the virus and some strains of staphylococci.

PART II: INVESTIGATIONS OF SYNERGISTIC ACTION OF STAPHYLOCOCCI AND VACCINIA.

Materials and Methods.

It was decided to use intracutaneous inoculations to study the results of mixed infections, to avoid secondary infection and to obtain accurate dosage. Also it was found possible to grind the whole thickness of skin in the intracutaneous lesions to assay virus content, which was not practicable when dealing with large scarified areas.

The results with 27 strains of staphylococci are tabulated, 7 of which were isolated from sheep pulp, 8 obtained from the National Collection of Type Cultures and 12 from Dr. Allison, of the Central Public Health Laboratory, Colindale. More sheep strains than those tabulated were in fact tested for their capacity to spread with vaccinia and for hyaluronidase production, but as they were not tested for haemolysin and fibrinolysin they are not included in the table. Their spread was proportional to their hyaluronidase production. The E.B.S. used in all these experiments was from a dried preparation known as 38/2, prepared by Dr. McClean. The rabbits were healthy adults of either sex.

Tests for spread of vaccinia.

The staphylococci were kept in Worth's medium until they were tested. They were inoculated into meat digest broth, pH 7·6, and incubated at 37° C. for 18 hours. The culture was spun down, the supernatant removed for the M.C.P. test (McClean, 1943), and the staphylococci washed three times, resuspended in saline to match the opacity of 100×10^6 *Salmonella typhi*.

Rows of intracutaneous inoculations were made on either side of clipped rabbits. In one row the inocula contained 0·1 ml. staphylococcal suspension and 0·1 ml. saline; in the other row, 0·1 ml. staphylococcal suspension and 0·1 ml. E.B.S. Two control inoculations of 0·1 ml. E.B.S. and 0·1 ml. saline were included. The lesions were traced on the third, fourth, and fifth days.

Some lesions were excised and sectioned for histological examination; others were minced and their virus content titrated. Some were allowed to take their natural course.

Tests for hyaluronidase production.

The supernatant fluids from the centrifuged broth cultures of the staphylococci were tested for hyaluronidase by the mucin clot prevention (M.C.P.) method as described by McClean (1943), except that horse serum was used instead of albumin in the preparation of the substrate-protein mixture. These tests were usually done on the same day as the inoculations, but when this was impossible they were stored at — 10° C. for not longer than a day or two before testing.

Strains were found to give remarkably constant titres of hyaluronidase when they were kept in 10 per cent glycerol broth, but their titres tended to decline after long storage in Worth's medium.

Test for coagulase production.

Broth cultures of the staphylococci were incubated at 37° C. with 1/10 human and rabbit plasma and the tubes read after 30 minutes and after overnight incubation.

Tests for fibrinolysin.

Sections of the intracutaneous lesions stained for fibrin showed that the fibrin meshwork was thinner and looser in the mixed infections where there was the greatest spread, so the possible influence of a bacterial fibrinolysin had to be considered in interpreting the enhancement of these lesions. A routine coagulase test was carried out using human and rabbit plasma in parallel. The tubes were sealed with wax and incubated for a week at 37° C. Twenty-three out of 27 strains coagulated rabbit plasma and none of these lysed the clot within a week, whereas with human plasma 14 out of 24 coagulase-positive strains subsequently lysed the clots, mostly about the fourth day.

Other experiments which have been briefly reported elsewhere (Lack, 1948) indicate that the staphylococcus produces an activator of human plasma protease (plasminogen or pro-fibrinolysin), but that lysis does not occur with rabbit fibrin because of an inhibitor (antiplasmin or antifibrinolysin) in rabbit plasma (Christensen, 1945). The staphylokinase titres of some strains are included in the table. To determine these, the staphylococci were grown by seeding into 10 ml. meat digest broth and shaking for 18 hours at 37° C., then spun down and the supernatant fluids dialysed under toluol against tap water in the cold for 48 hours. Serial fivefold dilutions of the residues were made by taking 0·2 ml. into 0·8 ml. saline. To each dilution were added 0·1 ml. 1 per cent human fibrinogen solution, 0·1 ml. 0·05 per cent human thrombin solution, and one drop of 0·5 per cent merthiolate.

In the control tube saline replaced the dialysed residue. All mixtures clotted to a firm gel within five minutes at 37° C. They were read in 18 hours, and the end point was taken to be the highest dilution of residue in which the clot was completely destroyed.

Haemolysin estimations.

These were carried out by a slight modification of Leonard and Holm's (1935) method. The staphylococci were first grown for 18 hours on blood agar. They were then subcultured in 10 ml. of the sloppy agar medium described, in 100 ml. flasks with cotton-wool plugs. The flasks were placed in large desiccators, the air evacuated and replaced by a mixture of 80 per cent O_2, 20 per cent CO_2, and the desiccators containing the flasks incubated at 37° C. for 48 hours. The contents of the flasks were then spun on an angle centrifuge at 1500 r.p.m. for an hour and the clear supernatants poured off. A drop of 0·5 per cent merthiolate was added and they were stored at — 10° C. until tested. Dilutions, starting with 1/5, 1/25 and subsequent twofold dilutions to 1/800 were made in gum saline; 0·5 ml. gum saline and 1 ml. 2 per cent rabbit red cell suspension were

added to each tube. The highest dilution of the supernatant to give complete haemolysis at the end of one hour is taken to be the end-point, and the dilution was expressed in terms of the final volume.

Experimental Results.

Tests for spread of vaccinia by staphylococci.

The results of one experiment on mixed infection in which staphylococci and vaccinia were inoculated separately and together into a rabbit's skin are reproduced in Fig. 1. Tracings of the lesions were made on the fifth day, then the

Staph. + E.B.S.	Staph. + saline.	M.C.P. titre.	Coagulase.	Haemolysin.	Titration of Virus * in lesion				
					1/100	1/1000	1/2T	1/4T	1/8T
		1/6400	+	1/300	c	10	5	2	1
		1/800	+	1/300	9	4	4	2	0
	Nil.	<1/200	—	1/20	8	6	3	0	0
Controls — E.B.S. + saline									
					3	3	1	0	0
					4	0	0	0	0

* Titration of virus in excised skin—ground skin applied in above dilutions to another rabbit and result recorded.

c = a confluent lesion. Number = number of pocks.

FIG. 1.—Spread of Vaccinia and Staphylococci when injected together.

rabbit was killed by air embolism, the lesions excised, frozen to — 10° C., sliced with a freezing microtome, ground with sand, and the pulp titrated for virus content on another rabbit. It will be seen that the combined staphylococcal and virus lesions are graded in size according to the hyaluronidase titre of the staphylococci. This occurred in all the mixed infections, whereas with the lesions produced by the same dose of the staphylococci alone there was not this same correlation. The supernatants and filtrates of the staphylococcal cultures also spread the virus in proportion to their hyaluronidase content.

Some strains of staphylococci which do not produce any lesion when injected with saline into the rabbit's skin, establish themselves when introduced in the

same quantity with vaccinia virus. Thus it appears that there is a synergistic action between staphylococci and vaccinia.

Intracutaneous lesions which were allowed to take a natural course usually formed abscesses in about a week which broke down, discharged their contents and healed in a few days. In one rabbit the abscesses caused by the mixed infection did not break down. After three weeks the rabbit was killed, the abscesses were dissected out and were found to grade in size according to the hyaluronidase production of their respective staphylococci. Staphylococci recovered from the pus were found to produce hyaluronidase to the same M.C.P. titres as before inoculation, and virus was recovered from the walls of the abscesses. There was no trace of the lesions produced by the virus alone or by the staphylococci alone.

Histological features of intracutaneous lesions.

Intracutaneous lesions which were excised for histological study were bisected through the site of inoculation, fixed in formol saline or mercuric chloride, and paraffin sections were stained with haematoxylin and eosin, Gram's method, Weigert's fibrin stain, Mann's methylene blue eosin and Giemsa's stain. Sections were examined from third-, fourth- and fifth-day lesions.

TABLE II.—*Characteristics of Staphylococci and their Synergistic Action with Vaccinia Virus in Rabbits.*

Staph.	Coagulase.		Lysis of coagulum.		Staphylokinase titre of dialysate.	Haemolysin titre.	Hyaluronidase titre.	Spread of virus.*
	Human.	Rabbit.	Human.	Rabbit.				
Heatley	+	+	—	—	..	1/400	1/25,600	5
6718 (1)	+	+	?	?	?	?	1/25,600	5
(2)	+	+	—	—	0	1/20	1/800	1·75
995	+	+	+	—	1/25	1/200	1/6400	4·8
996	+	+	—	—	..	1/400	1/3200	3
Humphrey	+	+	+	—	1/25	1/400	1/3200	3
993	+	+	—	—	..	1/800	1/3200	4
963	+	+	—	—	..	1/800	1/3200	3
7127	+	+	+	—	1/25	1/800	1/3200	4
962	+	+	+	—	1/25	1/1600	1/1600	3
999	+	+	+	—	1/25	1/1600	1/1600	4
7128	+	+	+	—	1/25	1/200	1/1600	2·25
2327	+	+	+	—	1/125	1/1600	1/800	2·5
2361	+	+	—	—	0	1/800	1/800	2·5
2386	+	+	+	—	0	1/20	1/800	3
2412	+	+	+	—	1/5	1/200	1/800	3
2436	+	+	+	—	1/5	1/800	1/800	*nil*
2721	+	+	—	—	0	1/800	1/800	„
Bett (1)	+	+	—	—	..	1/300	1/800	2·4
(2)	+	+	—	—	1/5	1/400	1/400	2
6832	+	+	+	—	1/125	1/1600	1/400	2
2404	+	+	—	—	1/5	1/800	1/400	2
2425	+	+	—	—	1/5	1/400	1/400	2
2449	+	+	—	—	1/5	1/200	1/400	*nil*
2387	+	+	+	—	1/3125	1/800	1/200	„
2444	+	+	+	—	1/5	1/200	1/400	2
961	—	—	N.C.	N.C.	0	1/20	<1/200	*nil*
Matt	—	—	„	„	0	1/20	<1/200	„
2408	+	—	+	„	1/5	1/800	<1/200	„

NOTE.—N.C. = No coagulum.

* Increase in area of combined lesion compared with that produced by E.B.S. alone.

Lesions produced by virus and saline showed a picture essentially similar to that described by Ledingham (1924). The cells of the Malpighian layer showed chromatolysis and swelling. The deeper portions of the hair follicles had disappeared in some areas and were being replaced by young vascular tissue. In the dermis the most striking feature was the proliferation of histiocytes over the skin muscle and throughout the perimysium. There were no haemorrhages, and in the central zone there were relatively few signs of inflammatory activity other than perivascular cuffing.

With staphylococci and saline the lesions varied from small discrete abscesses to a slight aggregation of polymorphs in the dermis.

With staphylococci and virus together the lesions varied very considerably both in extent and severity. The histological picture tallied with the macroscopic appearance. In those infections in which there was maximal spread, the hair follicles showed the characteristic changes of vaccinial infection over a wide area, extensive necrosis of the central zone of the dermis, many haemorrhages, and very heavy infiltration of the perimysium and skin muscle. Gram-stained sections showed the staphylococci to be dispersed throughout the lesion.

The results of the coagulase, mucin clot prevention and haemolysin tests are set out in Table II. The staphylokinase titres apply to fibrinolysis of human fibrin. It is possible to estimate similar activation of rabbit plasma protease by diluting out the inhibitor, but the work already done suggests that fibrinolysis is not a major factor in the enhancement of these mixed infections. The last column shows the increase in area of the mixed infection as compared with that produced by the same dose of virus alone in the same rabbit. It will be seen that the degree of spread is more closely correlated with the hyaluronidase titre than with haemolysin or fibrinolysin. The lesions produced by coagulase-positive staphylococci alone were always smaller than those produced by E.B.S. alone. The tracings of the staphylococcal lesions in Fig. 1 have been reduced to one third of the actual size. The coagulase-negative strains caused only a transient inflammation, which was no longer visible on the fifth day.

The action of vaccinia virus on phagocytosis of staphylococci by polymorphonuclear cells.

One other possibility was considered in trying to elucidate this synergistic action of staphylococci and vaccinia—that of interference with phagocytosis by the virus. The following experiment shows that this does not occur :

Two adult rabbits were given an intraperitoneal injection of 200 ml. sterile normal saline. Two similar rabbits were given the same volume of saline in which were suspended the contents of one ampoule of dried elementary bodies (E.B.S. 38/2). Four hours later 5 ml. of a washed suspension of staphylococcus strain "Humphrey" matched to 500×10^6 *S. typhi* were injected intraperitoneally into all four rabbits.

Half-an-hour later samples of peritoneal exudate were withdrawn and examined in a hot box. Phagocytosis was just as active in the exudates in which virus had been previously introduced as in the controls, and indeed the phygocytes remained active for a little longer in these exudates.

PART III: THE SYNERGISM OF VACCINIA AND CERTAIN STRAINS OF STREPTOCOCCI.

Rogers (1945) has shown that, unlike the staphylococcal enzyme, streptococcal hyaluronidase is adaptive, and that the M.C.P. titre of the strains he studied was proportional to the concentration of hyaluronate added to the culture medium when the pH was maintained at neutrality by the addition of 2·5 per cent sodium glycerophosphate. All the strains studied in these experiments were grown overnight in a medium composed of Evans' peptone broth 3·5 per cent; potassium hyaluronate 0·2 per cent; glucose 0·25 per cent; Na glycerophosphate 2·5 per cent; and the pH adjusted to pH 7·4. Tests for spread of vaccinia and for hyaluronidase were done in the same way as with staphylococci. The results are set out in Table III.

TABLE III.—*Synergistic Action of Streptococci and Vaccinia Virus in Rabbits.*

Group.	Type.	MCP titre.	Spread of combined lesion (*a*).	Spread of strep. alone (*b*).
A	1	0	*nil*	*nil*
	2	0	,,	,,
	3	0	1·5–3 *nil*	,,
	4	800	,,	,,
	4	0	,,	,,
	5	0	,,	,,
	6	25,600	6–14	,,
		400	3	,,
		0	*nil*	,,
	8	0	,,	,,
	9	0	,,	,,
	22	400	5	,,
	T4, 24, 29	200	*nil*	,,
	4 untyped strains	0	,,	,,
B	..	0	,,	,,
C	Untyped	0	1·5	,,
	,,	0	1·5	..
	2	1600	*nil*	..
	4	1600	2·5	..
	4	* 0–200	*nil*–2	,,
	4	* 0–800	,, –2·5	,,
	4	* 0–800	,, –2·5	,,
	7	102,400	14	,,
	20	0	2	,,
	21	800	1·7	,,
		* 0–200	*nil*–1·5	,,
D	..	0	*nil*	,,
G	28	* 0–1600	,,	,,

(*a*) Increase in area of combined lesion by comparison with that produced by virus alone.
(*b*) Lesions measured by comparison with area of original bleb.
* Before and after mouse passage.

It will be seen that only two strains, a Group A type 6 and a Group C type 7, produced a large amount of hyaluronidase *in vitro*, and with vaccinia virus these strains caused greatly enhanced lesions, four or five times larger than that produced by the virus alone. These two strains, when inoculated alone, caused insignificant lesions. Sections of these mixed infections showed widespread necrosis, which never occurred with either the streptococci or the virus alone.

The Group A type 6 is of particular interest, as some cultures in the above medium contained a large amount of hyaluronidase and others showed none at all. Dr. Nuala Crowley is now studying this strain, and has shown that on blood agar A.6 produces three morphological variants—mucoid, matt and glossy, all of which were type 6. The mucoid variants were capsulated. No individual colony, picked from a plate, produced hyaluronidase, though subculturing an inoculum of a large number of colonies into broth did sometimes produce hyaluronidase. Possibly hyaluronidase is produced by an intermittent variant which has not been isolated, and which responds to a medium containing plenty of substrate.

The Group C type 7 strain is the strain used by Rogers in his systematic study of the conditions controlling the production of hyaluronidase.

The Group A type 3 strain which never produced the enzyme nevertheless produced a moderate spreading lesion with virus in three rabbits. This strain is a very active producer of streptokinase. One of two Group A type 4 strains, on the other hand, did produce hyaluronidase *in vitro* but did not cause a spreading lesion with virus.

To determine whether enhancement of the viral lesion could be produced in an animal relatively resistant to vaccinia, guinea-pigs were used. In the guinea-pig, inoculations of E.B.S. into the dermis produce only a small papule 0·5 to 1 cm. in diameter, which disappears in three to four days. Five guinea-pigs were used, and the doses and dimensions of the resultant lesions are set out in Table IV. The streptococcus used in this experiment was the Group C type 7 strain obtained from Dr. Rogers.

TABLE IV.—*Synergistic Action of a Group C type 7 Streptococcus and Vaccinia Virus in the Guinea-pig.*

	Constituents of inoculum.				
	EBS 0·1 ml. + saline 0·1 ml.	Strep. susp. 0·1 ml. + saline 0·1 ml.	EBS 0·1 ml. + strep. susp. 0·1 ml.	Saline 0·1 ml. + supernatant 0·1 ml.	EBS 0·1 ml. + supernatant 0·1 ml.
Area of lesion:	mm.	mm.	mm.		mm.
Guinea-pig 1	5 × 5	3 × 3	10 × 20	Not tested	Not tested
„ 2	2 × 2	*nil*	10 × 14	„ „	„ „
„ 3	6 × 7	5 × 3	14 × 18	„ „	„ „
„ 4	3 × 3	*nil*	20 × 15	*nil*	5 × 7
„ 5	5 × 3	„	8 × 4	„	4 × 4

Lesions measured on fourth day.

Post-mortem examination of guinea-pig 2, which died on the fourth day, and guinea-pig 4, which was killed on the sixth day, showed that, whereas the lesions due to the virus alone and the streptococcus alone were barely visible, the lesion caused by the virus and streptococcus combined was a deep abscess extending into the spinal muscle. Histological examination confirmed the marked difference in the extent of the necrosis, and streptococci were found throughout the boundary zone of the abscess caused by the mixed infection, but were not to be seen anywhere in the lesion due to the streptococcus and saline inoculation. It will be noted that in the two guinea-pigs in which the E.B.S. was combined with the supernatant of a broth-culture of the streptococci there was enhancement of the viral lesion, whereas the supernatant alone produced only a transient erythema. Nine low or non-hyaluronidase-producing strains, belonging to Groups A, B, C, and D, did not produce any enhancement of vaccinial lesions in guinea-pigs.

DISCUSSION.

In the experiments described in Part I and II the two main observations to be explained are :

(1) vaccinia virus is spread and increased in proportion to the amount of hyaluronidase produced by a concomitant staphylococcal infection ;

(2) hyaluronidase-producing staphylococci which produce minimal lesions when injected alone are spread in proportion to their hyaluronidase production when injected with vaccinia virus.

Sprunt (1941) showed that the chance of a vaccinial lesion depends not only on the number of virus particles injected, but also on the number of susceptible host cells made available, and that spreading the virus over a larger area by using hyaluronidase increases the probability of a lesion. Enhancement of vaccinial lesions by staphylococcal hyaluronidase has been frequently reported, and has been confirmed in this laboratory ; it is not surprising that the enzyme produced *in situ* by staphylococci will do the same. Presumably the enzyme reduces the viscosity of the intercellular matrix, thereby enabling the virus particles to have access to a greater number of susceptible cells. Because these cells are the medium in which it multiplies, multiplication of the virus is, within limits, roughly proportional to the amount of hyaluronidase produced by the cocci. This would obviously not apply if very large amounts of this enzyme were produced, any more than it does if large amounts are injected, but it does appear to be the case within the range of enzyme production of these organisms.

With regard to the adjuvant action of these staphylococci on vaccinial lesions, it is interesting to recall an experiment recorded by Menkin (1936). He reported that the invasiveness of type III pneumococcus from the site of its cutaneous inoculation in rabbits may be delayed by the superimposed injection of a culture of *Staphylococcus pyogenes*. The " walling off " induced by the staphylococci so retarded the dissemination of the pneumococci that it allowed time for the local inflammatory reaction to dispose of them. Menkin maintained that the staphylococci caused such rapid plugging of the natural channels—the lymphatics and capillaries—by fibrin that the pneumococci were unable to get into the circulation. With mixed infections of staphylococci and vaccinia there is also " plugging " of these vessels ; the difference seems to be that the hyaluronidase-producing staphylococcus, while closing these roads, opens up alternative paths for the virus through the intercellular matrix.

The second observation is more difficult to explain. Why are the staphylococci themselves dispersed in proportion to their hyaluronidase production when they are introduced with vaccinia but not when they are injected alone ? It appears that the presence of the virus in the surrounding tissue prevents the " walling off " of the staphylococci, and allows them to follow in the wake of the virus which they themselves have enabled to spread. It would be interesting to know whether staphylococci produce coagulase more readily when introduced into healthy tissue, and whether they are more invasive when that tissue has already been damaged, as it is by infection with virus.

These observations support those of Böe (1944) that hyaluronidase production in *Staphylococcus aureus* is not correlated with virulence ; on the other hand, Schwabacher, Cunliffe, Williams, and Harper (1945) found that there was a

positive correlation of hyaluronidase, haemolysin and coagulase, and that none of the coagulase-negative haemolysin-negative strains in their series produced hyaluronidase, and our results are in agreement with theirs. This makes the assessment of the role of hyaluronidase in infection more difficult. An attempt has been made to immunize rabbits against staphylococcal hyaluronidase, but the enzyme, which was highly purified, proved to be a feeble antigen. If further attempts are successful a crucial experiment may be possible to establish the role of this enzyme, alone and in mixed infections.

Lesions produced by hyaluronidase-producing cocci and vaccinia virus developed more rapidly than the others. This may be because, as Duran-Reynals (1942) points out, this enzyme " acts on a pre-existing physiological barrier," whereas other bacterial weapons are directed " against barriers (leucocytosis, formation of fibrin clot, etc.) which the invader itself has called forth."

The synergistic action of streptococci and vaccinia virus appears to be more complex, for some strains have a spreading factor which is not hyaluronidase (Hobby, Dawson, Meyer and Chaffee, 1941). The fact remains that in this small series the two strains which produced the highest titres of this enzyme gave the maximal spread in combined lesions and were relatively innocuous when injected alone. The experiments with guinea-pigs recorded in Table IV may be compared with the results obtained by Hoffman (1931) He found that testicular extract containing hyaluronidase promotes the pathogenic action of the viruses of herpes, vesicular stomatitis of horses, Borna's disease and vaccinia, and that in some cases it allowed the viruses to infect resistant species or tissues.

Most of the recorded studies of the synergism of virus and bacteria are concerned with the influenza viruses (Shope, 1931 ; Glover, 1941 ; Wilson, Saslaw, Doan, Woolpert and Schwab, 1947). But Ledingham (1924) observed that neutralization of vaccinia virus with anti-vaccinial serum " stultified any action by contaminating cocci," and that " if the virus is rendered impotent the cocci play little or no part "

The enhancing action of hyaluronidase is not confined to viral infections ; McClean and Rogers (1943, 1944) have reported a marked increase in the lethal power of organisms of the gas gangrene group when mixed with hyaluronidase-producing staphylococci and streptococci, which were harmless by themselves. Both cocci and clostridia were rapidly disseminated throughout the body. The results recorded here suggest that hyaluronidase is an important factor in the synergism of vaccinia virus and Gram-positive cocci. This may throw some light on the well-known association of fulminating streptococcal and staphylococcal infections with smallpox.

SUMMARY.

Some strains of staphylococci and vaccinia virus have a synergistic action in the rabbit's skin, which is correlated with the hyaluronidase produced by the cocci rather than any other single factor examined.

In mixed streptococcal and virus infections hyaluronidase is one of the principal factors responsible for enhancement of the lesions, but lesions produced in the rabbit's skin by either staphylococci or streptococci alone are not proportional to hyaluronidase or any other single factor measured.

The significance of the synergistic action is discussed.

These observations explain the increased yield of pulp and virus due to the presence of cocci in routine production of vaccine lymph, and may shed some light on the fulminating streptococcal and staphylococcal infections associated with smallpox.

I wish to make grateful acknowledgment to Dr. McClean and Dr. Duthie of this Institute for their advice, and to Miss Proctor for technical assistance.

REFERENCES.

Bōe, J.—(1944) *Acta path. microbiol. scand.*, **21**, 587.
Christensen, L. R.—(1945) *J. gen. Physiol.*, **28**, 363.
Duran-Reynals, F.—(1928) *C. R. Soc. Biol. Paris*, **99**, 6.—(1942) *Bact. Rev.*, **6**, 197.
Glover, R. E.—(1941) *Brit. J. exp. Path.*, **22**, 98.
Hobby, G., Dawson, M., Meyer, K., and Chaffee, E.—(1941) *J. exp. Med.*, **73**, 309.
Hoffman, D. C.—(1931) *Ibid.*, **53**, 43.
Lack, C. H.—(1948) *Nature*, **161**, 559.
Ledingham, J. C. G.—(1924) *Brit. J. exp. Path.*, **5**, 332.
Leonard and Holm.—(1935) *J. Immunol.*, **29**, 209.
McClean, D.—(1930) *J. Path. Bact.*, **33**, 1045.—(1943) *Biochem. J.*, **37**, 169.
Idem and Rogers, H. J.—(1943) *Lancet*, i, 707.—(1944) *Ibid.*, ii, 433.
Menkin, V.—(1936) *J. infect. Dis.*, **58**, 81.
Meyer, K.—(1947) *Physiol. Rev.*, **27**, 335.
Rogers, H. J.—(1945) *Biochem. J.*, **39**, 435.
Schwabacher, Cunliffe, Williams and Harper.—(1945) *Brit. J. exp. Path.*, **26**, 124.
Shope, R. E.—(1931) *J. exp. Med.*, **54**, 373.
Sprunt, D.—(1941) *Ibid.*, **74**, 81.
Wilson, H. E., Saslaw, S., Doan, C. A., Woolpert, O. C., and Schwab, J. L.—(1947) *Ibid.*, **85**, 199.

ANTAGONIZATION OF THE ANTIBACTERIAL ACTION OF NEOARSPHENAMINE, PENICILLIN AND STREPTOMYCIN BY –SH COMPOUNDS.

R. D. SIMON.

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, London, W. 1.

Received for publication March 22, 1948.

The classical experiments of Fildes (1940) have shown that the antagonization by cysteine of the antibacterial action of mercuric chloride is due to direct combination between the –SH group and the mercury ion, but the mechanism of the antagonization by –SH of the antibacterial action of penicillin, streptomycin and arsphenamines has not apparently been determined, although antagonization by substances containing free –SH groups such as cysteine has been described (Albert, Falk and Rubbo, 1944; Cavallito and Bailey, 1944; Denkelwater, Cook and Tishler, 1945). The mechanism of this antagonization might be by interference with the drug's effect on bacterial metabolism, as in the antagonization of sulphonamides by p-aminobenzoic acid, or by direct combination between the drug and sulphydryl compound outside the bacteria.

In this communication experiments on the antagonizing action of cysteine on the antibacterial action of penicillin, streptomycin and arsphenamines are described, and from the results it is concluded that the mechanism is similar to that described by Fildes for mercuric chloride.

EXPERIMENTAL.

Materials.

–SH compounds.—The sulphur-containing compounds used were : *l*-cysteine-hydrochloride, *dl*-methionine, sodium thioglycollate and thiourea. Solutions were prepared in distilled water in a concentration of 1 in 50 (1 g. in 50 ml.) and the pH was adjusted to 7·6.

Arsphenamines.—The arsphenamines used were neoarsphenamine (sodium 3:3-diamino-4:4-dihydroxyarsenobenzene-N-methylene sulphoxylate), product Novarsenobillon (May & Baker, Ltd.) ; sulpharsphenamine (3:3-diamino-4:4-dihydroxyarsenobenzene-N:N-dimethylene sulphonate), product Sulfarsenol (Laboratoires de Biochimie Médicale, Paris) ; arsenoxide (3-amino-4-hydroxy-phenylarsenoxide), product Mapharside (Parke Davis, Ltd.) ; chlorphenarsine (3-amino-4-hydroxyphenyl-dichlorarsine hydrochloride) products Fontarsol (Société Parisienne d'expansion Chimique, Paris) and Halarsol (May & Baker, Ltd.).

Solutions of the arsphenamines were made in freshly boiled and cooled distilled water in concentrations of 1 in 50 (1 g. in 50 ml.) and were used after standing for 20 minutes, in order to standardize errors due to the ready oxidation of arsphenamines in solution.

Penicillin.—Solutions of the calcium salt of penicillin containing 100 and 10,000 units per ml. were made in sterile glass-distilled water, and stored at a temperature below 10° C.

Streptomycin.—A solution of the hydrochloride containing 10,000 microg./ml. was made up in sterile glass-distilled water, and stored at a temperature below 10° C.

Culture media.—Two culture media were used, the choice depending on whether the Oxford staphylococcus or *Bact. coli* (B.S.I. No. 148) was used as the test organism. The medium for the Oxford staphylococcus was 2 per cent peptone broth, with the addition of 2 per cent agar when a solid medium was required. With *Bact. coli* a synthetic culture medium was used adapted from the medium used by McIlwain (1941) in experiments on the reversal of the antagonization by acriflavine of *Bact. coli* and containing per 1000 ml. :

Potassium dihydrogen phosphate	4·5 g.
Ammonium chloride	0·5 g.
Ammonium sulphate	0·5 g.
Thiourea	0·5 g.
Ferrous ammonium sulphate (24 H_2O)	0·3 g.
Magnesium sulphate (7 H_2O)	0·001 g.
Lactic acid	1·9 ml.

This medium adjusted to pH 7·6 gave satisfactory growth of this strain of *Bact. coli*, whilst the sulph-hydryl compound thiourea was not in a sufficient concentration to affect the bacteriostatic concentration of the drugs used.

Methods.

In most of the experiments tubes containing mixtures of a bacteriostatic substance and a sulphur compound were prepared so that each series of tubes contained a constant concentration of one component and varying concentrations of the other. Serial two-fold dilutions of the variable component in broth, synthetic medium or distilled water were prepared to give 5/4 of the final concentration required and distributed in 0·8 ml. amounts in 3-in. × ½-in. test-tubes plugged with cotton-wool. To each tube of the experimental series was then added 0·2 ml. of the other component in the appropriate concentration. Control mixtures were included in each experiment, as is shown in the tables. When penicillin, neoarsphenamine and cysteine were tested together 0·1 ml. of a solution of each component was placed in the test-tube together with 0·7 ml. of medium. The final concentration of each component was thus 1/10 that of the original solution.

The bacteriostatic activity of the arsphenamines, streptomycin and penicillin in such mixtures, after incubation at 37° C. for intervals up to 24 hours were tested either by direct inoculation of mixtures in synthetic medium with *Bact. coli* or of mixtures in broth with staphylococci, followed by incubation at 37° C. overnight; or by the following plate method:

> Filter-paper discs of 10 mm. diameter were soaked in the solutions and then placed on the surface of poured plates of nutrient agar seeded with the Oxford staphylococcus. After overnight incubation at 37° C. the width of the zone of inhibition, that is, the intercept on the diameter between the filter paper disc and the edge of the zone of inhibition, was measured in mm. Three discs were usually placed on each plate, two from test solutions, and one which had been soaked in a solution containing a known concentration of penicillin or streptomycin.

The counteracting effect of iodine on the antagonizing power of cysteine was tested by estimating the bacteriostatic activity of the drug in mixtures of drug and cysteine which had been shaken with a solution of iodine in carbon tetrachloride until a permanent violet colour remained in the tetrachloride layer, a method which is described by Denkelwater, Cook and Tishler (1945) for reversal of the antagonizing effect of cysteine on the antibacterial action of streptomycin. An equal volume of iodine solution was sufficient for all except the highest concentrations of cysteine. Both the dilution method and the plate method were used to test bacteriostatic activity in these experiments.

Results.

Antagonization of the antibacterial action of arsphenamines.

A typical experiment showing the effect of sulphur compounds on the anti-bacteriostatic action of neoarsphenamine on *Bact. coli* in synthetic medium is shown in Table I. This table also shows that cysteine causes more profuse growth, as well as antagonizing neoarsphenamine. The results of a number of experiments with arsenical compounds using *Bact. coli* in the synthetic medium are given in Table II. It will be seen from this table that cysteine and sodium thioglycollate in a concentration of 1/500 are active against all the types of arsenical compounds tested, while methionine and thiourea are almost inactive.

TABLE I.—*The Effect of Various Types of Sulphur Compounds on the Bacteriostatic Action of Neoarsphenamine on* Bact. coli *in Synthetic Medium, Inoculated and Incubated Immediately after Mixing.*

Sulphur compound added to medium. Final concentration = 1/500.	Growth of *Bact. coli* in neoarsphenamine of concentration: Reciprocal × 10^{-3}.									
	2·5.	5·0.	10·0.	20·0.	40·0.	80·0.	160·0.	320·0.	640·0.	None.
None	−	−	−	−	−	−	−	+	+	+
Thiourea	−	−	−	−	−	−	−	+	+	+
Methionine	−	−	−	−	−	−	−	+	+	+
Sodium thioglycollate	+	+	+	+	+	+	+	+	+	+
Cysteine	−	+++	+++	+++	+++	+++	+++	+++	+++	+++

TABLE II.—*The Effect of Various Sulphur Compounds on the Bacteriostatic Action of Arsphenamines, Streptomycin and Penicillin on* Bact. coli *in Synthetic Medium Inoculated and Incubated Immediately after Mixing.*

Sulphur compound added to medium. Final concentration 2000 microg./ml. (1 in 500).	Concentration of bacteriostatic agent in mixture required to prevent growth of *Bact. coli* in microg./ml.					
	Neo-arsphenamine.	Sulph-arsphenamine.	Arsen-oxide.	Chlor-phenarsine.	Peni-cillin.	Strepto-mycin.
None	6·25	12·5	3·125	12·5	31	16
Thiourea	6·25	..	12·5	12·5	64	..
Methionine	6·25	50	6·25	12·5	31	..
Sodium thioglycollate	1600	50	50	400	64	..
Cysteine	400	400	100	400	1000	125

.. = Not done.

Against neoarsphenamine methionine has been found to be inactive up to a concentration of 1/64.

Further experiments with neoarsphenamine and cysteine have shown that there is only a limited range of concentrations of cysteine between 1/500 and 1/4000, which will antagonize the antibacterial action of neoarsphenamine in synthetic medium (Table III). The lower limit of this range is determined by

TABLE III.—*Relationship between the Molar Concentrations of Cysteine and Neoarsphenamine Resulting in the Antagonization of the Bacteriostatic Action of Neoarsphenamine on* Bact. coli *in Synthetic Medium Inoculated Immediately after Mixing.*

The minimum molecular ratio cysteine/neoarsphenamine resulting in growth is here 64/1.

Concentration of cysteine in mixture.		Growth of *Bact. coli* in neoarsphenamine of concentration: Reciprocal × 10^{-3}.							
Reciprocal × 10^{-3}.	Molar × 10^{-6}.	2·5.	5·0.	10·0.	20·0.	40·0.	80·0.	160·0.	None.
		Molar × 10^{-6}.							
		1000.	500.	250.	125.	62·5.	31·25.	15·6.	None.
0·25	32,000	−	−	−	−	−	−	−	−
0·5	16,000	−	−	+++	+++	+++	+++	+++	+++
1·0	8,000	−	−	−	+++	+++	+++	+++	+++
2·0	4,000	−	−	−	−	+++	+++	+++	+++
4·0	2,000	−	−	−	−	−	+	+	+
8·0	1,000	−	−	−	−	−	+	+	+
None	None	−	−	−	−	−	+	+	+

the fact that neoarsphenamine is not bacteriostatic below a concentration of 1 in 40,000, and the upper limit is due to the fact that cysteine inhibits growth of *Bact. coli* in synthetic medium above a concentration of 1 in 500. Table III also shows that the range of antagonization coincides with the range of cysteine concentrations in which growth is markedly stimulated. Within this range antagonization resulting in the growth of *Bact. coli* occurs only where the molecular ratio of cysteine to neoarsphenamine is greater than 32/1. This minimum ratio appears in Table III as 64 to 1 since two-fold dilutions are used, and it is calculated directly from the molar concentrations given in the table.

It has also been observed that in all tubes containing mixtures of cysteine and neoarsphenamine in molecular ratios of 32/1 and higher there is an absence of the brown or yellow colour which normally appears when neoarsphenamine in these concentrations is exposed to air, irrespective of the growth of *Bact. coli*. Moreover, tests with sodium nitroprusside in these mixtures show that free –SH is present in the tubes where the molecular ratio of cysteine to neoarsphenamine is 16/1 or more, which includes all tubes in which antagonization results in growth.

The results of these antagonization experiments are not affected by varying the time of incubation before the addition of neoarsphenamine, cysteine, or inoculum to the other components, provided that neoarsphenamine is added before visible growth of *Bact. coli* has occurred.

Experiments of a similar type to that shown in Table III, with arsenoxide, have shown that a constant molecular ratio of cysteine to arsenoxide of between 32/1 and 16/1 is necessary before antagonization of the action of arsenoxide on *Bact. coli* can be observed in the synthetic medium. Sodium nitroprusside tests have shown that free –SH groups are present in mixtures where the molecular ratio is 8/1 or more, and the presence of –SH has been found to prevent the formation of brown colour due to oxidation in tubes containing mixtures of arsenoxide and cysteine with molecular ratios greater than 8/1.

Antagonization of the antibacterial action of penicillin.

In the case of penicillin the only –SH compound of those tested that has been found to be an active antagonizer at a concentration of 1/500 is cysteine (Table II). Sodium thioglycollate, which is active against arsenical compounds, has no apparent effect on penicillin even in a concentration of 1 in 100, equivalent to a molecular ratio of thioglycollate to penicillin of 1000/1.

The results of a number of experiments on the antagonization of penicillin by cysteine are summarized in Table IV. This table shows that with a 600-fold increase in the concentration of penicillin (40 to 25,000 units per ml.) there is only a 4-fold increase in the concentration of cysteine necessary to antagonize the action of penicillin on staphylococci, and consequently the molecular ratio of cysteine to penicillin necessary for antagonization decreases from 100/1 to 1/1·5. It will be noted that the decrease in the ratio of cysteine required with increasing concentrations of penicillin, together with the use of the plate method of testing the activity of penicillin against staphylococci instead of against *Bact. coli* in synthetic medium, has made it possible to obtain antagonization of all concentrations of penicillin with sub-bacteriostatic concentrations of cysteine, so that the complication of inhibition of growth by cysteine encountered in

TABLE IV.—*The Relationship between the Molar Concentrations of Cysteine and Penicillin in Broth resulting in Antagonization of the Antibacterial Action of Penicillin. Antagonization is shown by Growth of Staphylococcus in the Fluid Medium, Inoculated after 3 Hours' Preliminary Incubation of the Mixture of Penicillin and Cysteine in Broth.*

Final concentration of cysteine in mixture.		Growth of staphylococcus in penicillin of concentration: Units per ml.				
		40.	400.	6400.	25,000.	None.
				Molar × 10^{-4}.		
1 in—	Molar × 10^{-4}.	0·8.	8·0.	128.	500.	None.
100	800	+	+	+	+	+
250	320	+	+	+	+	+
500	160	+	+	+	−	+
1000	80	+	+	−	−	+
2000	40	−	−	−	−	+
10,000	8·0	−	−	−	−	+
None	None	−	−	−	−	+

testing the arsenical compound has not arisen. In the mixtures in Table IV free –SH can be detected by the nitro-prusside test in all tubes where cysteine has been added in a concentration of 1/100 irrespective of the concentration of penicillin, but with lower concentrations of cysteine free –SH can be detected only when the penicillin concentration is 40 units per ml. It is therefore apparently not necessary for antagonization to have free –SH present in the mixture in sufficient excess to be detectable by the nitroprusside test. In experiments with *Bact. coli* in synthetic medium it has been found that a molecular ratio of cysteine to penicillin of 10/1 is necessary to antagonize the action of penicillin in a concentration of 400 units per ml. This ratio is of the same order as the ratio necessary to antagonize the action of this concentration of penicillin on staphylococci in broth.

When parallel series of mixtures of penicillin and cysteine are made in broth and in distilled water it has been found that the capacity of cysteine to antagonize penicillin is four times less in broth than in aqueous solution. This protecting action of broth is clearly shown in Table V, in which the activity of penicillin is measured by the plate method. It will be seen that under the conditions of this experiment penicillin in the presence of cysteine loses its bacteriostatic power in 3 hours in distilled water but not in broth. It is also shown that broth protects

TABLE V.—*The Effect of Broth and Distilled Water on the Antagonizing Action of Cysteine on the Bacteriostatic Action of Penicillin, Tested by the Plate Method after Preliminary Incubation for 0, 1 and 3 Hours.*

Final concentrations.	Width of zone of inhibition of staphylococcus in mm. produced by filter-paper discs soaked in solutions in—					
	Broth after incubation for (hours)			*Distilled water* after incubation for (hours)		
	0.	1.	3.	0.	1.	3.
Penicillin, 10 units/ml.	16	16	16	16	16	17
„ 10 units/ml. 1/2000 cysteine	16	16	16	16	8	0
„ 10 units/ml. 1/2000 HCl	16	15	16	16	0	2
Controls:						
1/2000 cysteine	0	0	2	0	0	0
1/2000 HCl	2	0	0	0	0	0

penicillin against the action of hydrochloric acid in a concentration of 1/1000 (pH 3). The results with cysteine, however, are not affected by pH, and in any case, as has been mentioned, the cysteine hydrochloride solutions, normally at pH 3, have always been adjusted to pH 7·6.

It is remarkable that there is no apparent reaction between cysteine and penicillin in the filter-paper discs and agar plates used in testing the process of antagonization in mixtures of cysteine and penicillin. This can be seen in Table V, and noticeably in Table VIII, where the zone of inhibition of staphylococcus formed by a mixture of penicillin and cysteine is markedly decreased by a 30-minute increase in the time of preliminary incubation. This difference in the size of the zone of inhibition is observed after the plates have been incubated for 18 hours, which would be more than sufficient time for antagonization to be complete. The cessation of antagonization may be due to rapid diffusion of the penicillin and cysteine through the agar. This diffusion would cause dilution of the penicillin and cysteine, and the minimum molecular ratio of cysteine to penicillin necessary for antagonization would consequently be raised above the ratio of the concentrations actually present in the medium.

Antagonization in mixtures of neoarsphenamine, penicillin and cysteine.

The neoarsphenamine and penicillin antagonization experiments have shown that there is a definite minimum ratio of cysteine to drug molecules below which antagonization does not occur. This suggests that in a mixed solution of penicillin and neoarsphenamine only one drug would be antagonized by the addition of a limited amount of cysteine in a concentration giving the correct molecular ratio for the antagonization of either drug, especially if drug and cysteine formed a stable complex. Experiments were therefore designed to show whether both drugs could be antagonized in a mixture containing only sufficient cysteine to antagonize one. The concentrations of penicillin and neoarsphenamine used for these experiments were chosen, so that either drug alone was sufficient to inhibit the growth of the test staphylococcus in broth and each was antagonized by the same concentration of cysteine, the penicillin only being present in sufficient concentration to produce a zone of inhibition in the plate test. Suitable concentrations for this purpose are 40 units of penicillin per ml. neoarsphenamine 1 in 1000, and cysteine 1 in 500, giving a molecular ratio of penicillin/cysteine/neoarsphenamine of 1/200/30. Neoarsphenamine was added to the mixture either immediately or after preliminary incubation of the two other components at 37° C. for various times. The mixture of the three components was incubated at 37° C. and then tested at 0, 1 and 3 hours for bacteriostatic action on the staphylococcus by the plate method and by direct inoculation, broth being added to the mixtures prepared in water.

A typical experiment in which all three components have been mixed immediately before incubation is shown in Table VI. The results of inoculation of the staphylococci directly into the mixtures, not shown in Table VI, have confirmed that the concentration of cysteine was sufficient to antagonize either drug separately, but not both together. The results of the plate tests, given in Table VI, have been confirmed by several other experiments, and show that in broth solution penicillin is antagonized by cysteine to a greater extent in the presence of neoarsphenamine than in its absence, whereas in water, the addition

TABLE VI.—*The Effect of Neoarsphenamine and Cysteine on the Bacteriostatic Activity of Penicillin in Broth and Water Solutions, tested by the Formation of a Zone of Inhibition of Growth on Nutrient Agar Plates.*

Final concentrations of substances tested.	Width of zone of inhibition in mm. formed by filter-paper soaked in solution in—					
	Broth after incubation for (hours)			*Water* after incubation for (hours)		
	0.	1.	3.	0.	1.	3.
Penicillin, 40 units/ml.	16	15	4	16	16	15
,, 40 units/ml. + cysteine 1/500	15	4	6	15	6	0
,, 40 units/ml. + cysteine 1/500 + neoarsphenamine 1/1000	14	1	0	15	9	9
Controls :						
Penicillin 40 units/ml. + neoarsphenamine 1/1000	16	14	14	15	15	15
Neoarsphenamine 1/1000	0	0	0	0	0	0
Cysteine 1/500	0	0	0	0	0	0
Neoarsphenamine 1/1000 + cysteine 1/500	0	0	0	0	0	0

of neoarsphenamine greatly reduces the antagonization of penicillin by cysteine. This effect has also been observed in experiments using concentrations of the three components giving varying molecular ratios both above and below the minimum necessary for antagonization by cysteine.

Antagonization of the antibacterial action of streptomycin by cysteine.

A typical experiment to test the antagonizing effect of cysteine on the antibacterial action of various concentrations of streptomycin is shown in Table VII.

TABLE VII.—*Relationship between the Molar Concentrations of Cysteine and Streptomycin Resulting in the Antagonization of the Bacteriostatic Action of Streptomycin on* Bact. coli *in Synthetic Medium.*

The minimum molecular ratio cysteine/streptomycin resulting in growth is here 128/1.

Concentration of cysteine in mixture.		Growth of *Bact. coli* in streptomycin of concentration : Microg./ml.								
		1,000.	500.	250.	125.	62·5.	31.	16.	8.	None.
Microg./ml.	Molar × 10^{-6}.	Molar × 10^{-6}.								
		2000.	1000.	500.	250.	125.	62·5.	31.	16.	None.
10	80,000	−	−	−	−	−	−	−	−	−
4	32,000	−	−	−	+	+	+	+	±	±
2	16,000	−	−	−	−	+	+	+	+	+
1	8,000	−	−	−	−	−	+	+	+	+
None	None	−	−	−	−	−	−	−	+	++

This table also shows that there is only a short range of concentrations of streptomycin in which the activity of cysteine can be observed under these conditions. The lower limit is set by the concentration of 16 microg./ml. necessary to antagonize the growth of *Bact. coli* in synthetic medium, while the higher limit of the range is fixed by the high molecular ratio of cysteine/streptomycin required for antagonization combined with the bacteriostatic action of high concentrations of cysteine.

It can be seen from Table VII that with all the concentrations of cysteine

tested a molecular ratio of cysteine to streptomycin of at least 128/1 is necessary before antagonization is observed by the growth of *Bact. coli*. Tests with sodium nitroprusside have been positive only in series with cysteine 10 microg./ml. Insufficient –SH to give a positive test remained in the tubes of the other series irrespective of the growth of *Bact. coli*.

Reversal by iodine of the antagonizing action of cysteine.

Mixtures of penicillin, neoarsphenamine or streptomycin with cysteine were shaken with a solution of iodine in carbon tetrachloride until no further decolorization was observed, and the bacteriostatic power of the mixture was then tested. The results of such an experiment in aqueous solution are given in Table VIII. This table shows that shaking with iodine did not cause reversal

TABLE VIII.—*The Effect of Iodine on the Antagonization by Cysteine of the Bacteriostatic Action of Penicillin and Streptomycin in Aqueous Solution.*

Solution final concentration.	Zone of inhibition of Oxford staphylococcus in mm. formed by filter-paper disc soaked in solutions after incubation for (hours)					
	0.	½.	1.	3.	4.	5.
Penicillin, 40 units/ml.	13	13	13	14	13	13
,, cysteine 1/250	12	9	3	0	0	0
,, cysteine shaken with I_2	12	6	0	0	0	0
Streptomycin, 4000 microgm./ml.	14	14	15	13	13	12
,, cysteine 1/250	12	9	12	8	7	6
,, cysteine shaken with I_2	12	12	15	12	11	12

of the antagonization by cysteine of the antibacterial action of penicillin, but even an increase in the rate of antagonization. This effect may be due to the partition coefficient of penicillin between water and carbon tetrachloride reducing the concentration of penicillin in the water.

On the other hand, Table VIII shows that the antagonization of the antibacterial action of streptomycin by cysteine, which is incomplete in the molecular ratio of cysteine to streptomycin here used, was reversed when the mixture even after 5 hours' incubation was shaken with iodine solution, and a large zone of inhibition of the staphylococcus on the plate was again formed.

TABLE IX.—*The Effect of Shaking with Iodine Solution in Carbon Tetrachloride on the Antagonization by Cysteine of the Antibacterial Action of Neoarsphenamine on* Bact. coli *in Synthetic Medium.*

Solution added.	Growth of *Bact. coli* in the mixture diluted with synthetic medium to a concentration of 1 in—							
	2.	4.	8.	16.	32.	64.	128.	None.
	Neoarsphenamine of theoretical concentration: Reciprocal × 10^{-3}.							
	2·5.	5.	10.	20.	40.	80.	160.	None.
Nil	–	–	–	–	–	–	++	++
I_2 in CCl_4	–	–	–	–	–	+	+	+
Cysteine 1 in 25:								
After 0 hrs. incubation	–	–	++	++	++	++	++	++
,, 3 ,, ,,	–	–	–	+	++	++	++	+
Cysteine 1 in 25 and I_2 in CCl:								
After 0 hrs. incubation	–	–	–	+	++	++	++	++
3 ,, ,,	–	–	++	++	++	++	++	+

It was not possible to use the plate method when the drug was neoarsphenamine because of the small zone of inhibition formed, so that mixtures were tested by inoculating *Bact. coli* into serial two-fold dilutions of the mixture in the synthetic medium. These tubes were then incubated overnight before reading the results. Table IX shows that in this case shaking with iodine did not reverse the antagonization of neoarsphenamine by cysteine unless the mixture was shaken with iodine immediately after the cysteine was added, so that some cysteine was removed before it could affect the neoarsphenamine, when a very slight reversal of the antagonization was obtained. This result, however, may be due to a time factor, since the iodine-cysteine reaction is much slower than the neoarsphenamine-cysteine reaction.

DISCUSSION.

The experiments described here have shown that *l*-cysteine, which contains one –SH group, is an active antagonizer of the antibacterial action of penicillin, streptomycin and arsphenamines, provided that the molecular ratio of cysteine to drug is not less than a certain value which is constant for neoarsphenamine, arsenoxide, and streptomycin in the appropriate conditions, but varies with different concentrations of penicillin.

The minimum molecular ratio of cysteine to drug necessary for the antagonization of the action of neoarsphenamine on *Bact. coli* in a synthetic medium is between 32/1 and 64/1. If we assume with Eagle (1939) that neoarsphenamine splits at the As=As bond, and so can combine with 4 –SH groups, the number of cysteine molecules necessary for antagonization of neoarsphenamine is between 8 and 16 times the theoretical number required for combination. This is in agreement with the value of 10 times the theoretical amount, calculated by Eagle from his experiments on the antagonization of the antispirochaetal action of arsphenamines by cysteine. The constancy and importance of these ratios has also been shown by experiments made under the same conditions with arsenoxide, the molecule of which contains only one arsenic atom. The molecular ratio of cysteine to arsenoxide required for antagonization of the antibacterial action is between 16/1 and 32/1, but this is also between 8 and 16 times the theoretical amount of cysteine necessary, since one arsenoxide molecule can combine only with 2 –SH groups.

It can be presumed that the arsphenamine and cysteine in these mixtures combine by direct reaction between the arsenic ion and the –SH group. The constant molecular ratio, the prevention of the formation of oxidation colour in the solutions of arsphenamine, the disappearance of free –SH, the inability of iodine solutions to reverse the antagonization effect of the cysteine, and the failure of a limited amount of cysteine to antagonize both neoarsphenamine and penicillin, although sufficient for either drug alone, all point to combination between cysteine and arsphenamine, the compound formed being stable and not bacteriostatic. The mechanism of antagonization of the antibacterial action of arsphenamines by cysteine (and other –SH compounds by analogy) is therefore presumably disappearance of the active arsphenamine from solution. This mechanism is similar to that for the antagonization of the antispirochaetal action of arsphenamine (Eagle, 1939), and is also similar to the mechanism

described by Fildes (1940) for the antagonization of the antibacterial action of mercuric chloride by –SH compounds.

Voegtlin (1925) and Eagle (1939) have suggested that trypanosomes and spirochaetes are attacked by arsphenamines through the –SH groups in their proteins, and Fildes (1940) has put forward the theory that mercuric chloride attacks bacteria by combining with the –SH groups of the bacterial protein and so interfering with an enzyme system. Since the antibacterial action of arsphenamines is antagonized by combination of the arsphenamine with –SH, it seems likely, by analogy with Fildes' theory, that the antibacterial action of the arsphenamines is due to combination with –SH groups in bacterial proteins.

The mechanism of the antagonization of penicillin by cysteine results in certain differences in experimental results from those obtained with arsphenamines. The most important differences found in these experiments, apart from the fact that sodium thioglycollate appears to have no antagonizing power, is that no constant molecular ratio is obtained when cysteine antagonizes the antibacterial action of penicillin under conditions similar to those of the arsphenamine experiments.

As shown in Table IV there is a constant minimum molecular ratio of cysteine to penicillin necessary for antagonization of each concentration of penicillin, but this ratio decreases as the concentration of penicillin increases, until with high concentrations of penicillin a ratio representing fewer molecules of cysteine than of penicillin is sufficient for antagonization.

These results are qualitatively in agreement with those of Cavallito, Bailey, Haskell, McCormick and Warner (1945), who at the same time found that sodium thioglycollate did not easily antagonize penicillin. But it is not made clear by these workers what medium was used for their experiments. The importance of the medium in which the antagonization of the action of penicillin by cysteine is observed has been shown by the experiments described here. It has been found that broth appears to have a protecting action (Table V), since no antagonization of penicillin is obtained with concentrations of cysteine which completely antagonize the activity of penicillin in aqueous solution. This effect was apparently not due to stabilization of the pH, since the same results were obtained at pH 3·0.

The effect of altering the medium is also shown in the results obtained when cysteine was added to mixtures of penicillin and neoarsphenamine (Table VI). A concentration of cysteine sufficient to antagonize the action of either penicillin or neoarsphenamine caused in broth solution total inactivation of only the penicillin, whilst in aqueous solution the penicillin was only partly antagonized. It was therefore not possible to test the activity of the neoarsphenamine in this mixture, as this could only be done by direct inoculation after dilution with broth.

The results of all the experiments described here, including the variable molecular ratio of cysteine to penicillin necessary for antagonization, the disappearance of free –SH in high concentrations of penicillin, the inability of a limited amount of cysteine to antagonize both penicillin and neoarsphenamine, the failure of iodine solution to reverse antagonization by cysteine and the gradual increase in antagonization shown in mixtures of penicillin and cysteine being tested by the plate method, suggest that the antagonization of the antibacterial action of penicillin by cysteine must be due to some type of reaction between

the two substances rather than to interference with bacterial metabolism by penicillin counteracted by the action of cysteine, as suggested by Shwartzmann (1946) for the antagonizing action of methionine and other –S–S-compounds on penicillin.

The reaction between penicillin and cysteine, since penicillin contains no "heavy metal," is probably of the oxidation-reduction type. The equilibrium of such a reaction could be altered by changing the solvent for example from distilled water to broth. Oxidation-reduction reactions are readily undergone by –SH compounds, which also easily combine with α–β unsaturated ketones, and since the penicillin molecule may contain a similar ketonic structure, the cysteine may combine with this group. A similar mechanism is suggested for the antagonization of the antibacterial action of certain quinones by –SH compounds by Colwell and McCall (1946). It is significant that these workers found that quinones were protected from cysteine by broth, as the experiments described here have shown penicillin to be protected. Addition of neoarsphenamine to penicillin-cysteine system, however, has been found to reduce the protective action of broth. This supports the oxidation-reduction theory, since the interference by neoarsphenamine is probably due to the ease with which neoarsphenamine can be oxidized, so that the equilibrium in broth is altered and becomes nearer that in water, resulting in total antagonization of the penicillin. In an aqueous solution, on the other hand, the neoarsphenamine may be less readily oxidized, but combines with the cysteine, thus reducing the cysteine available to antagonize the penicillin.

It is probable that the combination between penicillin and cysteine is only the first stage in the antagonization of the antibacterial action of penicillin by cysteine, and that this combination is followed by disintegration of the penicillin molecule, liberating a substituted cysteine compound of the penicillamine type (Report of Committee on Medical Research, Washington, and M.R.C. London, 1945), which can then start a chain reaction by combining with more penicillin. In support of this view is the fact that low concentrations of penicillin need relatively more cysteine to antagonize their antibacterial action than do high concentrations, in some of which a less than equimolecular concentration of cysteine is sufficient. Moreover, concentrated solutions of penicillin are less stable, and the antibacterial activity falls off more rapidly than in low concentrations in the absence of added antagonizers, which suggests an autocatalytic chain-reaction degradation, the rate depending on the concentration of the reactants.

This suggested chain-reaction mechanism is also consistent with the finding that shaking with iodine does not reverse the antagonization of penicillin by cysteine, because it is only at the start of antagonization that cysteine would be important, and this reaction between cysteine and penicillin takes place very rapidly, while the iodine-cysteine reaction is slow.

The antagonization of the antibacterial action of streptomycin by cysteine is different from both penicillin and neoarsphenamine antagonization. In this case there is a constant minimum molecular ratio of cysteine to streptomycin necessary for antagonization, and free –SH is only present when high concentrations of cysteine are added (Table VII), but antagonization of the antibacterial action of streptomycin by cysteine is completely reversed by shaking with iodine solution, and broth does not appear to have any protecting action. The constant

molecular ratio and the disappearance of free –SH suggest that antagonization of streptomycin by cysteine has a mechanism similar to the –SH antagonization of the arsphenamines, and there is direct combination between the cysteine and streptomycin.

The very high molecular ratio of cysteine to streptomycin required before antagonization is observed suggests that each molecule of streptomycin must combine with at least two –SH groups before complete antagonization of the antibacterial action is observed. This reaction under the usual experimental conditions tends apparently towards formation of a compound, resulting in antagonization, but when shaking with iodine reduces the concentration of cysteine the balance of the reaction is altered in favour of the two separate components and probably almost the whole of the streptomycin is set free, so that antibacterial activity is again observed in the mixture.

The mechanisms of antagonization of the antibacterial action of the three compounds, neoarsphenamine, penicillin and streptomycin by cysteine, discussed here, appear to be variations of one of the basic principles of antagonization. This principle is interference with the antibacterial activity of a compound by blocking the group or groups by means of which the drug attacks the bacteria. This is well shown by neoarsphenamine, in which the arsenic ion forms a stable compound with the –SH group of cysteine, and there is no further antibacterial power. Streptomycin also is antagonized by combining with cysteine, but the streptomycin cysteine complex, like other organic complexes, is dependent for existence on a balance of concentrations which can be destroyed, for example, by shaking the mixture with iodine, and thus reducing the –SH concentration. Penicillin shows a different type of variation, since after initial combination with cysteine the penicillin molecule probably breaks down into –SH compounds, initiating a chain-reaction degradation of the penicillin, which results in irreversible antagonization of antibacterial action.

SUMMARY.

The antagonization of the antibacterial action of penicillin, streptomycin and arsphenamines by cysteine is described. This antagonization has been observed by inoculating bacteria into mixtures of cysteine and drug in broth and synthetic media, and also by placing filter-paper discs soaked in the mixtures on seeded agar plates.

Antagonization occurs when there is a molecular ratio between the drug and cysteine, constantly 1 to 64 for neoarsphenamine, 1 to 128 for streptomycin and variable for penicillin, under the experimental conditions.

It is concluded that cysteine antagonizes arsphenamines, streptomycin and penicillin by direct combination between the –SH group and the drugs, outside the bacteria. In the case of streptomycin it is suggested that a complex is formed in a reaction which can be reversed by reducing the concentration of cysteine present, while in the case of penicillin it is suggested that the antagonization is assisted by the development of a chain reaction due to breakdown of penicillin molecules into substituted cysteines.

REFERENCES.

Albert, A., Falk, J. E., and Rubbo, S. D.—(1944) *Nature*, **153**, 712.
Cavallito, C. J., and Bailey, J. H.—(1944) *Science*, **100**, 390.

Iidem, HASKELL, T. H., MCCORMICK, J. R., AND WARNER, W. F.—(1945) *J. Bact.*, **50**, 61.
COLWELL, C. A., AND MCCALL, M.—(1946) *Ibid.*, **51**, 659.
DENKELWATER, R., COOK, M. A., AND TISHLER, M.—(1945) *Science*, **102**, 12.
EAGLE, H.—(1939) *J. Pharmacol.*, **66**, 436.
FILDES, P.—(1940) *Brit. J. exp. Path.*, **21**, 67.
MCILWAIN, H.—(1941) *Biochem. J.*, **12**, 1311.
Report of Committee on Medical Research, Washington, and Medical Research Council, London.—(1945) *Nature*, **156**, 766.
SHWARTZMANN, G.—(1946) *J. exp. Med.*, **83**, 65.
VOEGTLIN, C.—(1925) *Physiol. Rev.*, **5**, 63.

THE EFFECT OF STROMA-FREE HAEMOGLOBIN ON THE ISCHAEMIC KIDNEY OF THE RABBIT.

A. W. BADENOCH AND E. M. DARMADY.

From the R.A.F. Hospital, Wroughton.

Received for publication March 22, 1948.

IN a previous communication (Badenoch and Darmady, 1947) renal lesions produced by partial occlusion of the renal artery in the rabbit were reported, which were comparable in many ways with those seen in human traumatic uraemia and in cortical necrosis of the kidney. The experiments confirmed the view that the kidney is susceptible to anoxia, but it did not explain why such kidney failure is so frequently associated with transfusion reactions, crushing injury, blackwater fever, etc. This new investigation was therefore planned in conjunction with the previous research to determine what part, if any, stroma-free haemoglobin might play in the production of this syndrome.

HISTORICAL.

Investigators in this field have perhaps shown a greater failure to confirm each others' results than in other branches of medicine, and although the history of incompatible blood transfusion begins as early as 1667 (Denys quoted by G. Keynes, 1922), serious work did not start until Levy (1904) discussed the whole subject and carried out preliminary investigations. This was followed by the experiments and observations of Barratt and Yorke (1909), Yorke and Nauss (1911), and Baker and Dodds (1925), and these have had a considerable effect upon subsequent investigators. These workers using rabbits produced haemoglobinurea, and found that kidney lesion occurred more commonly in the animal with acidosis. They concluded that precipitation of haemoglobin in the presence of an acid urine caused death by obstruction of tubules, and that the remedy was to maintain an alkaline urine. Later observers gave contradictory evidence as to which of the haemoglobin derivatives were responsible, although they all

confirmed that to produce the lesion it was necessary that these should be in acidosis. Thus De Gowin, Osterhagen and Anderson (1937), and De Gowin, Warner and Randall (1938), found that in dogs haemoglobin could produce anuria, and Anderson, Morrison and Williams (1942) produced kidney failure with ferrihaematin. Later Bywaters and Stead (1944) claimed that myohaemoglobin was responsible, but this was disputed by Bing (1944), who, unable to confirm these results, suggested that methaemoglobin alone was responsible. This conflicting evidence has been criticized by Foy, Altman, Barnes and Kondi (1943), who have suggested some other factor might be responsible. They state that the acidotic rabbit is dehydrated, and that therefore the animal is liable to develop uraemia in any case. Their conclusions were partially based on the work of Gersh (1940), who injected pigs' haemoglobin into the rabbit and found that cast formation was greater in the dehydrated animal. Foy, Altman, Barnes and Kondi (1943) also suggested that the substance used (ammonium chloride) to produce acidosis might be equally responsible for the lesion produced, and this was subsequently confirmed by Govan and Parkes (1946), who showed that acidosis produced by this agent leads to degeneration of the kidney epithelium. On the other hand, Bayliss (1920), working with rabbits, held that haemoglobin in itself is not toxic, and suggested that if kidney failure occurred it was due to the "globin" portion producing an anaphylactic reaction. Later the clinical and experimental work of Bordley (1931), Otternburg and Fox (1938), De Navasquez (1940) and Yuile (1942) established that stroma-free haemoglobin is not in itself toxic to man or animals. This is confirmed by reports of a more clinical nature, where it is found that haemoglobin is freely excreted in blackwater fever without uraemia occurring (Stephens, 1937), and that when uraemia does supervene it occurs equally in acidosis or alkalosis or with a normal alkali reserve (Wakeman, Morrell, Eisenman, Sprint and Peters, 1932; Foy and Kondi, 1941; Maegraith and Findlay, 1944). These findings are in agreement with our own results, in which we found that myohaemoglobin was frequently excreted in the severely wounded casualty without uraemia occurring. This has suggested that before fatal haemoglobinuria can be produced it is necessary that the kidney first be damaged. This has been supported by Yuile, Gold and Hinds (1945), who have shown experimentally that when the kidney is injured by sub-lethal doses of sodium tartrate and by partial occlusion of the renal vessels, the added introduction of stroma-free haemoglobin causes renal changes similar to the transfusion kidney and blackwater fever. This was originally suggested by Bing (1944), and is supported by the work of Corcoran and Page (1945). More recent studies by Harrison, Bunting, Ordway and Albrink (1947) on dogs after exposure to arsine and following the injection of methaemoglobin showed that renal injury could only be demonstrated in animals with extreme haemolysis, and when the renal blood-flow was greatly reduced.

Somewhat similar investigations carried out by Corcoran and Page (1945) have shown that in the rat subjected to tourniquet shock and injected intravenously with meta-myohaemoglobin, renal changes developed similar to crush anuria. They had previously established that each of the procedures of intravenous injection of meta-myohaemoglobin and tourniquet shock were not alone lethal, and concluded that renal damage is apparently produced in human beings by a combination of a vasoconstrictive renal ischaemia, aciduria, and the deposition in the kidneys of the muscle pigment myohaemoglobin.

METHOD OF INVESTIGATION.

General procedure and dietetic measures.

As in the previous investigation, the rabbits used in these experiments were obtained from the animal farm at the Experimental Station, Porton; their weight varied between 1·8 and 3·2 kilograms. The general procedure and dietetic measures were as previously outlined, since it was felt essential in this experiment, too, to ensure an alkaline urine. The degree of urea retention was assessed by blood urea levels by the method described. A description of the operative procedure has already been given, although in this series sodium pentothal was used as an anaesthetic. The operative procedure consisted of a right nephrectomy, followed by temporary partial occlusion of the left renal artery. Following removal of the arterial clamp, 10 ml. of stroma-free haemoglobin was injected into the marginal ear vein. This dose was selected because it was thought to be comparable with that usually fatal in the human. In the latter the production or injection of 500 ml. of haemolysed blood is universally fatal, and it was calculated that 500 ml. would correspond to 10 ml. in a rabbit.

Preparation of the stroma-free haemoglobin and serum.

For the first ten animals used in this investigation the preparation of a stroma-free haemoglobin differed from that used in the later series. For the former 10 ml. of blood was withdrawn from the marginal ear vein one week before operation, and allowed to run into graduated sterile glass bottles containing 1 ml. 3 per cent sodium citrate. This blood was centrifuged at 3000 r.p.m. and the supernatant fluid withdrawn. The erythrocytes were washed with three changes of sterile saline and made up to 10 ml. with pyrogen-free water. Haemolysis was completed by freezing to — 20° C., and thawing three times by means of an acetone CO_2 snow mixture. The resulting fluid was centrifuged at 16,000 to 20,000 r.p.m. for 20 minutes, and the supernatant fluid transferred to a sterile container and stored at 2–4° C. until required for use. For the remainder of the animals the haemoglobin was prepared in bulk. For this purpose four normal adult rabbits were anaesthetized, a sterile cannula was placed in the carotid artery, and the animals were bled out into sterile 3 per cent sodium citrate. The blood was mixed and, with aseptic precautions, washed three times with sterile saline, and finally made up to its original volume with pyrogen-free sterile water. This solution was then haemolysed in the manner previously described, and centrifuged at 16,000 to 20,000 r.p.m. for 20 minutes. The resulting supernatant fluid was withdrawn under sterile precautions, and stored in bottles each containing 10 ml. until required for use. To ensure that the fluid was sterile and free from stroma it was examined microscopically and cultured aerobically and anaerobically. As a photo-electric colorimeter was not at that time available it was not possible to standardize the concentration of these solutions, but as the blood had been made up to its original volume and a standard dose was used for each animal, this was not felt to interfere with the subsequent result.

For the preparation of the serum a similar procedure was used, except that the blood from two normal rabbits was allowed to clot. Before use the serum was centrifuged at 6000 r.p.m. to remove erythrocytes, and cultured. It was stored in 10 ml. quantities until ready for use.

Description and result of experiment.

Before a full description of the experiment is given, it is well to examine the effect of the operative procedure and the stroma-free haemoglobin on the mortality rate, but as in a previous communication we showed that right nephrectomy with manipulation of the left kidney did not cause any fatal result, this was not repeated. In this series, therefore, an operation as previously described was performed without the closure of a clamp, followed by the intravenous injection of 10 ml. of stroma-free haemoglobin at the conclusion of the operation. Six animals were used in these series, two of which received their own prepared haemoglobin, and there were no deaths. As a further control, 9 animals were subjected to a right nephrectomy, and the left artery occluded for half-an-hour. Subsequently 10 ml. of stroma-free haemoglobin was injected; again there were no deaths. This confirmed that neither the operation nor the stroma-free haemoglobin were in themselves lethal. These results are shown in Table I and Table II.

TABLE I.—*Control Series.*

Description of experiment.	Mortality rate.	Number of animals.
Operation without closure of the clamp	0	10
Operation without closure of the clamp + stroma-free haemoglobin	0	6*
½-hour occlusion + stroma-free haemoglobin	0	9

* Two animals received their own haemoglobin.

Previously we showed by our technique that the period of clamping and the mortality rate were in proportion to each other, and that the mortality rate for a 60-minute occlusion was 20 per cent, those for 90 minutes was 60 per cent and for 120 minutes 100 per cent. The results are summarized in Table III.

TABLE III.

Period of occlusion.	Mortality rate.	Number of animals.
Operative procedure only	0%	10
60 minutes	20%	10
90 ,,	60%	10
120 ,,	100%	10

The remainder of the experiments were therefore directed towards the effect of stroma-free haemoglobin on animals subjected to a right nephrectomy from 60-minute and 90-minute occlusion. Twelve animals were subjected to a 60-minute occlusion with the injection of stroma-free haemoglobin. Mortality rate from this procedure was found to be 16·6 per cent, as compared with mortality rate of 20 per cent by occlusion only. It must be stated, however, 3 animals, of which 2 died, received their own prepared haemoglobin. It was concluded that the injection of the haemoglobin in this series did not contribute an insurmountable burden to the damaged kidney. Further details and biochemical investigations are shown in Table IV and V.

TABLE II.—*Serial Blood Urea Levels (mg. per 100 c.c.) in 15 Control Animals, after Injection of 10 ml. of Stroma-free Haemoglobin.*

BLOOD UREA LEVELS.

Rabbit No.	Pre-operative.	Days after operation. 1	2	4	5	6	9	10	11	12	13	14	15	18	19	20	23	25	26	28	Remarks.
77	46	..	32	68	..	55	..	45	..	(killed)	..	..	..	..	..	..	..	..	..	..	Received own haemoglobin.
79	34	..	24	56	..	..	..	53.	..	(killed)	..	..	..	..	..	..	..	..	..	..	
104	46	..	66	..	42	..	..	..	36	..	..	..	..	..	..	31	..	..	..	28	(killed) ..
111	43	..	45	..	37	..	58	..	..	..	38	..	..	..	..	..	..	32	(killed)	..	. ..
123	28	..	38	..	38	..	..	..	36	..	..	..	..	30	..	..	..	25	(killed)	..	. ..
128	39	..	56	..	47	..	42	..	72	..	..	..	..	..	34	..	..	29	(killed)	..	. ..
97	42	..	146	..	42	..	..	..	38	..	..	..	(killed)	..	..	..	..	..	..	..	. ..
100	33	..	98	..	36	..	..	..	49	..	..	..	..	..	..	..	..	..	(killed)	..	. ..
101	28	..	108	..	36	..	..	..	42	..	..	..	..	..	..	..	..	..	(killed)	..	. ..
113	33	156	128	..	51	..	58	..	37	..	..	..	56	..	..	..	44	(killed)	..	..	. ..
114	31	110	60	..	42	..	54	..	31	..	..	..	..	..	..	..	(killed)	..	..	..	. ..
115	37	120	63	..	70	..	90	..	38	..	..	..	38	..	..	..	36	(killed)	..	..	. ..
116	27	..	195	..	152	..	48	..	..	..	34	..	..	..	..	..	..	28	(killed)	..	. ..
117	25	..	84	..	..	..	..	70	..	..	..	40	..	31	..	..	..	..	33	(killed)	. ..
127	34	..	43	..	60	..	42	..	..	..	28	..	..	..	..	33	..	..	30	(killed)	. ..

(6 received nephrectomy without clamping; 9 received nephrectomy with 30 minutes' occlusion of left renal artery.)

TABLE V.—*Serial Blood Urea Levels (mg. per 100 c.c.) in 12 Rabbits Receiving 60 Minutes' Occlusion of the Left Renal Artery and Right Nephrectomy Followed by an Injection of 10 c.c. of Stroma-free Haemoglobin.*

BLOOD UREA LEVELS.

Rabbit No.	Pre-operative.	Days after operation. 2	3	4	5	8	9	10	12	13	15	17	20	21	22	23	25	26	27	Remarks.
69	48	480	(died)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	Received own haemoglobin.
70	44	470	(died)	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	..	
72	40	164	..	40	..	..	..	59	(killed)	..	..	..	..	..	..	..	..	..	..	
107	40	264	..	..	160	58	..	..	36	..	..	..	..	..	..	..	41	(killed)	..	
108	29	344	..	..	336	36	..	..	68	..	..	..	..	..	..	..	37	(killed)	..	. ..
109	32	520	..	..	256	208	..	..	53	..	..	62	..	..	..	..	59	(killed)	..	. ..
110	24	118	..	..	62	44	..	..	..	..	..	..	..	(killed)	..	..	..	..	..	. ..
126	31	208	..	..	146	..	46	..	..	72	..	..	39	..	..	..	..	30	(killed)	. ..
129	29	368	..	..	63	..	78	..	..	..	..	..	..	(killed)	..	..	..	..	..	. ..
130	26	148	..	130	..	68	..	..	..	..	27	..	..	30	(killed)	..	..	..	..	. ..
131	22	98	..	132	..	70	..	..	..	..	40	..	..	56	(killed)	..	..	..	..	. ..
132	34	172	..	142	..	70	..	..	..	..	30	..	..	34	(killed)	..	..	..	..	. ..

TABLE IV.—*Period of Occlusion 60 Minutes.*

Description of experiment.	Mortality rate.	Number of animals.
Occlusion only . . .	20% (2)	10
Occlusion + stroma-free haemoglobin	16·6% (2)	12*

* Three animals, of which two died, received own haemoglobin.

The interest of this experiment lies in the effect of the stroma-free haemoglobin consequent upon a 90-minute occlusion, the results of which are summarized in Table VI.

TABLE VI.—*Summary of Experiments Involving Animals with a Right Nephrectomy and Occlusion of the Left Artery for 90 Minutes.*

Description of experiment.	Mortality rate.	Number of animals.
Occlusion only	60% (6)	10
Occlusion + stroma-free haemoglobin added	83% (10)	12*
Occlusion + serum added . .	70% (7)	10

* Five animals which received their own haemoglobin died.

In 10 animals subjected to clamping only the mortality rate was 60 per cent, whereas with the addition of serum in the same number of animals the mortality rate was 70 per cent. When, however, stroma-free haemoglobin is introduced at the end of the operative procedure, the mortality rises from 60 per cent to 83 per cent, 12 animals being used, of which 10 were unable to recover. It must be pointed out, however, that of 5 animals which received their own haemoglobin, all died. Biochemical details are shown on Table VII and VIII.

TABLE VII.—*Serial Blood Urea Levels (mg. per 100 c.c.) in Rabbits Receiving 90 Minutes' Occlusion of the Left Renal Artery and Right Nephrectomy Followed by an Injection of 10 ml. of Stroma-free Haemoglobin.*

BLOOD UREA LEVELS.

Rabbit No.	Pre-operative.	Days after operation. 2	3	4	5	6	7	8	9	13	14	21	22	23	Remarks.
36	52	..	304	..	..	300	..	(died)	..	..	..	..	..	..	Received own haemoglobin.
70	44	480	(died)	..	..	..	..	..	..	..	..	..	..	..	
74	29	368	(died)	..	..	..	..	..	..	..	..	..	..	..	
75	25	544	(died)	..	..	..	..	..	..	..	..	..	..	..	
80	40	464	..	(died)	..	..	..	..	..	..	..	..	..	..	
118	34	184	..	..	448	..	..	..	264	(died)	..	..	..	..	..
119	36	380	..	..	800	(died)	..	..	..	..	..	..	..	..	..
120	27	(died)	..	..	..	..	..	..	..	..	..	..	..	..	..
121	25	480	..	..	200	..	..	..	42	68	..	31	(killed)	..	..
122	48	180	..	..	896	..	..	..	(died)		..	..	..	..	..
124	28	240	..	..	166	(died)	..	..	..	..	..	..	..	..	..
125	33	240	..	..	496	..	..	..	80	66	..	24	(killed)	..	..

TABLE VIII.—*Serial Blood Urea Levels (mg. per 100 c.c.) in 10 Animals Receiving 90 Minutes' Occlusion of the Left Renal Artery and Right Nephrectomy Followed by an Injection of 10 ml. of Rabbit Serum.*

Rabbit No.	Pre-operative.	Blood Urea Levels. Days after operation. 2	3	4	6	7	8	9	11	12	15	21	28	Remarks.
135	36	360	..	448	..	768	(died)	..	..	..	..	..	..	..
136	29	220	..	262	..	248	..	..	..	..	47	34	45	(killed)
137	35	278	..	208	..	(died)	..	..	..	..	..	..	..	..
138	28	244	298	(died)	..	..	..	..	..	..	..	..	..	..
139	30	208	(died)	..	..	..	..	..	..	..	..	..	..	..
140	27	216	(died)	..	..	..	..	..	..	..	..	..	..	..
141	25	116	..	262	..	(died)	..	..	..	..	..	..	..	..
142	32	111	..	416	..	42	..	..	..	..	33	27	40	(killed)
143	26	204	..	200	..	496	..	..	(died)	..	..	..	..	..
144	38	220	..	448	..	216	..	..	..	..	72	32	47	(killed)

On more careful examination of Table VIII further points of interest are seen. For it shows not only that the mortality rate is increased in those animals receiving the haemoglobin solution, but also that the period of survival is diminished.

The survival rates from these three groups of animals with 90 minutes' occlusion are summarized on Table IX.

TABLE IX.—*Summary of Experiments Showing Survival Rates in Animals Receiving 90 Minutes' Occlusion.*

Description of experiment.	Number of animals.	Days of survival. 1–3.	4–7.	8–15.	Survived.
Stroma-free haemoglobin added	12	4	3	3	2
Serum added	10	1	4	2	3
Clamping only	10	2	2	2	4

From these experiments it is concluded that the added haemoglobin plays a significant part in the severity and mortality of the illness.

DISCUSSION.

In the past not only has there been considerable controversy as to whether haemoglobin *per se* is nephrotoxic, but also its preparation has been the subject of much criticism. Many discrepancies have been due to the lack of standardization in the production of haemoglobin solutions. Care has therefore been taken to ensure, firstly, that an aseptic technique was used throughout and that the solutions were sterile; secondly, that absolute haemolysis was achieved and that the rupture of the erythrocytes was complete; thirdly, that the stroma was completely removed by centrifugalization, this being confirmed by microscopic examination; fourthly, that the solutions administered were isotonic; fifthly, that the solutions were standardized as a haemoglobin content. Unfortunately, photo-electric colorimeters were not available at the time so that standardization of the solutions could not be checked, and where haemoglobin solutions were self donated it is possible that a variation existed. In the remainder, however,

the bulk preparation and constant dose (10 millimetres) suggests that the dosage of haemoglobin was constant. Spectroscopic examinations confirmed that the haemoglobin solution was composed of oxyhaemoglobin.

These experiments confirm the findings of Bordley (1931), Ottenburg and Fox (1938), De Navasquez (1940) and Yuile (1942) that haemoglobin *per se* is not toxic. They also go further, and indicate that damage to the kidney is essential before a detrimental effect can occur from haemoglobin or its derivatives. This substantiates the work of Yuile *et al.* (1945), Corcoran and Page (1945). It seems, however, that our experiments have a greater implication. For they suggest that unless a critical degree of renal damage is achieved, haemoglobin or pigment does not seem to have an adverse effect. This seems to explain why haemoglobinuria frequently occurs both in blackwater fever and in casualties without death occurring, although it is known that minor degrees of kidney damage may be present (Maegraith and Findlay, 1944 ; Darmady, 1947).

In a similar sort of way, it is possible that discrepancies noticed in the experiment with haemoglobin solutions in acidotic animals may be explained by our results, for Govan and Parkes (1946) have shown that ammonium chloride will result in degeneration of the renal epithelium, and that the degree of damage could be varied by the dosage administered. It is possible that in the previous investigations there was a variation of dosage which resulted in a variation in the degree of renal damage produced. On the other hand, Bing (1944) and Harrison *et al.* (1947) have shown that methaemoglobin is relatively more toxic than haemoglobin, and this may play a secondary part.

These experiments may, however, be criticized because of the increased mortality rate from animals receiving their own haemoglobin. It is therefore proposed to repeat these experiments using a standardized dose of haemoglobin, and by producing ischaemia and haemolysis of blood by other means. These investigations may explain why in uraemia with crush syndrome and blackwater fever the prognosis is so poor, and further, why the incidence of fatal uraemia is so common in cases which develop intra-vascular haemolysis. They do not explain why or how the initial renal injury occurs, and although many interesting theories exist which suggest that stroma is responsible, it is felt that further investigations are necessary.

Our thanks are due to the Air Ministry for permission to publish this work, and to Dr. R. G. MacFarlane, of the Radcliffe Infirmary, Oxford, for help in preparing the haemoglobin solutions.

REFERENCES.

ANDERSON, W. A. D., MORRISON, D. B., AND WILLIAMS, E. F. J.—(1942) *Arch. Path.*, **33**, 589.
BADENOCH, A. W., AND DARMADY, E. M.—(1947) *J. Path. Bact.*, **59**, 79.
BAKER, S. L., AND DODDS, E. C.—(1925) *Brit. J. exp. Path.*, **6**, 247.
BARRATT, J. O. W., AND YORKE, W.—(1909) *Ann. trop. Med. Parasit.*, **3**, 1.
BAYLISS, W. M.—(1920) *Brit. J. exp. Path.*, **1**, 1.
BING, R. J.—(1944) *Johns Hopk. Hosp. Bull.*, **74**, 161.
BORDLEY, F.—(1931) *Arch. intern. Med.*, **47**, 288.
BYWATERS, E. G. L., AND STEAD, J. K.—(1944) *Quart. J. exp. Physiol.*, **33**, 53.
CORCORAN, A. C., AND PAGE, I. H.—(1945) *Texas Rep. Biol. Med.*, **3**, 528.
DARMADY, E. M.—(1947) *Brit. J. Surg.*, **34**, 262.

De Gowin, E. L., Osterhagen, H. F., and Anderson, M.—(1937) *Arch. intern. Med.*, **59**, 432.
Idem, Warner, E. L., and Randall, W. L.—(1938) *Ibid.*, **61**, 609.
De Navasquez, S.—(1940) *J. Path. Bact.*, **51**, 413.
Foy, H., and Kondi, A.—(1941) *Trans. Roy. Soc. trop. Med. Hyg.*, **34**, 343.
Idem, Altman, A., Barnes, H. D., and Kondi, A.—(1943) *Ibid.*, **36**, 197.
Gersh, I.—(1940) *Anat. Rec.*, **65**, 371.
Govan, A. D. T., and Parkes, J.—(1946) *J. Path. Bact.*, **58**, 505.
Harrison, H. E., Bunting, H., Ordway, N. K., Albrink, W. S.—(1947) *J. exp. Med.*, **86**, 339.
Keynes, G.—(1922) ' Blood Transfusion.' London (Oxford University Press).
Levy, L.—(1904) *Dtsch. klin. Med.*, **81**, 359.
Maegraith, B. G., and Findlay, G. M.—(1944) *Lancet*, ii, 403.
Otternburg, R., and Fox, C. L.—(1938) *Amer. J. Physiol.*, **123**, 516.
Stephens, J. W. W.—(1937) ' Blackwater Fever.' Liverpool University Press.
Wakeman, A. M., Morrell, C. A., Eisenman, A. J., Sprint, D. L., and Peters, J. P.—(1932) *Amer. J. trop. Med.*, **12**, 407.
Yorke, W., and Nauss, R. W.—(1911) *Ann. trop. Med. Parasit.*, **5**, 287.
Yuile, C. L.—(1942) *Physiol. Rev.*, **22**, 19.
Idem, Gold, M. A., and Hinds, A. G.—(1945) *J. exp. Med.*, **82**, 361.

THE RELATIONSHIP BETWEEN NITRATASE AND TETRATHIONASE ADAPTATION AND CELL GROWTH.

M. R. POLLOCK* and S. D. WAINWRIGHT.†

From the Medical Research Council Unit for Bacterial Chemistry, Lister Institute, London, S.W. 1.

Received for publication April 14, 1948.

It has previously been shown (Knox and Pollock, 1944 ; Pollock, 1946) that the rate of reduction of nitrate or tetrathionate by washed suspensions of certain coliform bacteria could be increased very considerably, without any evidence of cell growth, simply by incubation with a suitable H-donator (such as mannitol or glucose) in the presence of the specific substrate. There is some evidence (Spiegelman, Reiner and Morgan, 1947) for regarding such enzymatic adaptation as the formation or modification of specific enzyme protein, but in the report of the work which follows this problem has not been further investigated and adaptation has been considered purely functionally, as a specific increase in the ability of a suspension of cells to catalyse a particular chemical reaction.

It seemed of importance to determine the effect upon enzyme adaptation of altering conditions, by the addition of an assimilable form of N, such that generalized cell growth and multiplication could occur ; in other words, to study

* Leverhulme Research Fellow.
† Leverhulme Research Scholar.

the adaptation of tetrathionase and nitratase during the early stages of growth in a nitrate and tetrathionate medium respectively.

The results of these experiments showed that nitratase adaptation preceded increase in cell mass ; and that later, after growth had begun, the rate of adaptation was very much greater than the rate of increase of the total bacterial protoplasm. This led to an attempt to discover whether the extent of previous adaptation might have any determining influence on the initiation or speed of cell growth in a medium containing the specific substrate.

Monod (1942) has brought strong suggestive evidence, by his work on bacterial " diauxie," that specific adaptation to the oxidation of certain carbohydrates is a necessary preliminary to growth on these substrates as sole source of energy and carbon. The idea of adaptation or " reorientation " of enzymes during the lag phase is one which forms the basis for the theoretical discussions of Hinshelwood (1944, 1946) on bacterial growth. So far, however, there has been little direct biochemical evidence supporting this concept. The main difficulty in all attempts to follow metabolism during the early growth phases is to obtain an initial concentration of cells small enough to be capable of considerable increase during the course of the experiment, yet sufficiently large to produce a concentration of metabolites high enough to be accurately estimated. Methods for the estimation of thiosulphate are not sensitive enough for this purpose ; and combined growth and adaptation experiments have had to be confined to nitratase where use of the Griess Ilosvay reagent enables concentrations of nitrite as low as 2×10^{-5} M to be measured with reasonable accuracy.

METHODS.

Nitrate and tetrathionate reduction were followed in evacuated Thunberg tubes, the nitrite and thiosulphate being estimated as previously described (Pollock, 1946 ; Pollock and Knox, 1943).

The organism used was a Gram-negative coliform bacterium of the " Intermediate " group, called " 1433 " (Pollock, 1946).

Cell suspensions of " 1433 " were obtained from the growth on tryptic meat agar in Roux bottles incubated aërobically at 35° for 16 hours. The cells were twice washed with distilled water and made up to standard strength by opacity measurements on the Spekker photo-electric absorptiometer.

Growth was followed in specially selected 6-in. $\times$ $\frac{3}{4}$-in. stoppered tubes, fitted with vacuum taps, filled with argon (O_2 content less than 0·001 per cent). These were incubated at 35°, and the growth estimated directly on the Spekker absorptiometer by means of a specially made tube adapter, modelled on that described by Tinsley and Pizer (1945). A standard opacity curve for this estimation was prepared by plotting dry weights of organisms against drum readings (log Io/I). Results on the growth curves, however, are recorded directly as log Io/I.

Nitratase and tetrathionase activities were determined under conditions where adaptation does not occur, by using formate as H-donator (Pollock, 1946), and expressed as μ mols. nitrite or thiosulphate formed per mg. dry weight per hour.

O_2 uptake was followed in Warburg manometers at 35° with M/5 phosphate +M/100 glucose, and NaOH in the centre cups ; final volume 2·5 ml.

Medium for growth experiments consisted, unless otherwise stated, of M/100 glucose, M/10 phosphate buffer pH 7·2, M/600 $MgSO_4$, M/100 NH_4Cl and M/100 sod. nitrate or tetrathionate as H-acceptor.

Inoculum for growth experiments corresponded to an initial concentration of approximately 50 μg. dry weight of cells/ml.

Nitratase adaptation experiments were done at 35° in evacuated Thunberg tubes, and, unless otherwise stated, in a basal mixture consisting of M/100 glucose, M/5 phosphate buffer pH 7·2, and M/100 sod. nitrate with a concentration of cells of approximately 200 μg./ml.

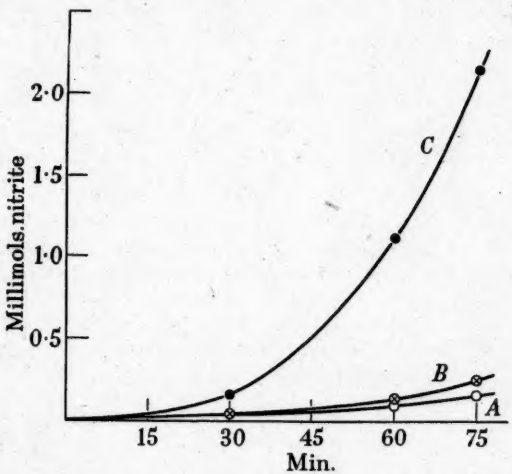

FIG. 1.—Effect of external source of N on nitratase adaptation.
A, Control (no additional N).
B, + M/100 NH_4Cl.
C, + M/4000 each of 18 amino-acids.

The adaptation curves have been plotted directly as nitrite concentration against time. As previously pointed out (Pollock, 1945) such curves are a composite picture of the effect of the conditions under test on both adaptation and rate of enzyme action. There is, also, the possibility of a direct effect upon the enzyme of some metabolite produced during the course of the reaction. In order to obviate these possible complications, samples of cells have in most cases been removed and washed, after adaptation under different conditions, and their nitratase activities measured as described above.

RESULTS.

The effect on adaptation of addition of external N source.

Fig. 1 shows nitratase adaptation curves: (A) with no additional source of N; (B) with M/100 NH_4Cl; and (C) with M/4000 each of 18 mixed amino-acids (see Appendix (i)) in the presence of M/600 $MgSO_4$. The periods recorded cover the stages before there was more than a 15 per cent increase in opacity

readings due to the beginning of growth. The pronounced stimulatory effect of the amino-acids in contrast to the slight effect of NH_3 is striking. Nitratase activity measurements on washed samples of cells after 75 minutes' adaptation (Table I) showed nearly 3½ times more active nitratase in cells treated with amino-acids in comparison with those adapted without an external source of N.

TABLE I.—*Effect of Additional N Source on the Rate of Nitratase Adaptation.*

	Nitratase activity (μ Mols. nitrite/mg./hr.).
Before adaptation	0·12
After 75 min. adaptation :	
No addition (control)	1·02
+ M/100 NH_4Cl	1·10
+ 18 amino-acids (M/4000 of each) . .	3·44

All tubes contained M/5 phosphate, M/100 glucose, M/100 sod. nitrate and 0·2 mg./ml. cells.

Varying the concentration of amino-acids between M/8000 and M/2000 did not affect the rate of adaptation, but reduction of the number of amino-acids added caused an obvious drop in the speed of adaptation (even though the total

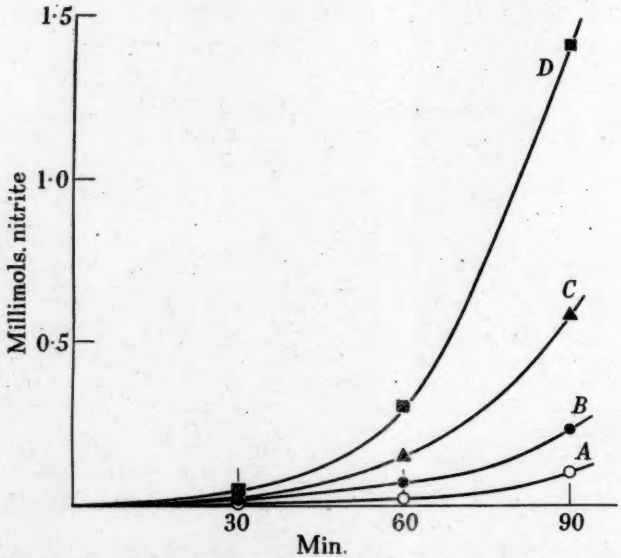

FIG. 2.—Effect of number of added amino-acids on nitratase adaptation.

A, Control (none added).
B, + M/800 each of alanine, glutamic acid, proline and leucine.
C, + M/1600 each of above 4 + lysine, aspartic acid, serine and valine.
D, + M/4000 each of all 18 amino-acids.

amino-acid N was kept constant by increasing the concentration of the remaining acids) roughly proportional to the number omitted. Fig. 2 and Table II show

TABLE II.—*Effect of Number of Amino-acids Added on the Rate of Nitratase Adaptation.*

	Nitratase activity (μ Mols. nitrite/mg./hr.).
Before adaptation	0·23
After 90 min. adaptation :	
Control (no addition)	0·65
+ 4 amino-acids* (M/800 of each)	0·78
+ 8 amino-acids* (M/1600 of each)	1·38
+ 18 amino-acids* (M/4000 of each)	2·90

* For composition see text and Appendix (i).

All tubes contained M/5 phosphate, M/100 glucose, M/100 sod. nitrate and 0·2 mg./ml. cells.

the effect of adding 4 amino-acids (M/800 each of alanine, glutamic acid, proline and leucine), 8 amino-acids (M/1600 each of the previous four mentioned plus lysine, aspartic acid, serine and valine) and M/4000 each of all 18 amino-acids. The actual concentration of additional N added was thus roughly the same. It was also found that, with the possible exception of proline, tryptophan, tyrosine and phenylalanine the omission of any single amino-acid caused a detectable drop in the speed of adaptation.

Further addition of 16 growth factors (see Appendix (ii)) was found to have no significant effect.

Effect of Mg^{++} on adaptation.

Erratic results were at first obtained when testing these growth factors, until it was discovered that certain solutions acquired pronounced activity after "Seitz" filtration, and that a powerful adaptation-promoter was leached out of the filter pads by solutions at acid or neutral pH. The factor was eventually identified as Mg^{++}. Washings of Ford filter pads with distilled water (pH 5·2) gave a solution with maximal stimulation of adaptation and strong positive tests with *p*-nitrobenzene-azo-α-naphthol; and it was possible completely to replace the effect by a solution of M/600 $MgCl_2$ or $MgSO_4$. Detectable stimulatory effects were apparent at a concentration of 0·15 μg. Mg^{++}/ml. However, this action of Mg^{++} was not so pronounced (*a*) if the concentration of cells used was increased about 10 times, giving a final concentration of 2 mg./ml. instead of the usual 200 μg./ml.; (*b*) if broth was used as a source of amino-acids instead of a mixture of synthetic amino-acids, or (*c*) if the phosphate buffer concentration was reduced from M/5 to M/10. Fig. 3 and Table III show that the discrepancy between broth and synthetic amino-acids is reduced in the presence of Mg^{++}, and the same is true of the differences between adaptation rates in the two concentrations of phosphate (Fig. 4 and Table IV). The marked falling off in the rate of adaptation per cell with dilution of the suspension has been referred

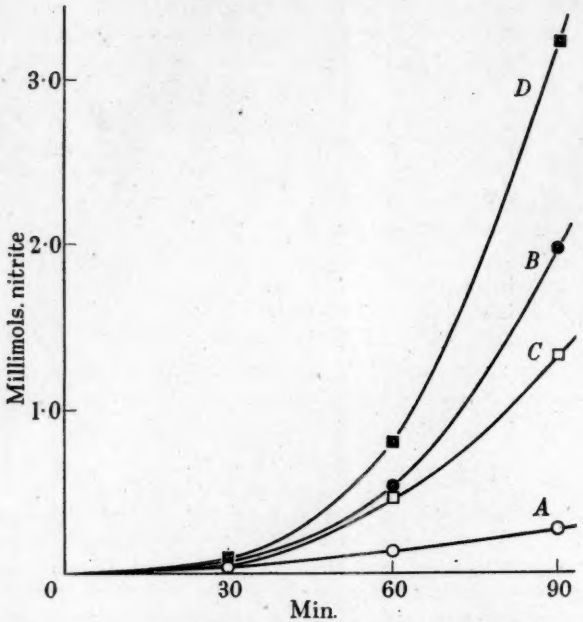

FIG. 3.—Effect of Mg^{++} on nitratase adaptation in the presence of broth and amino-acids.

A, + M/4000 each of 18 amino-acids.
B, ,, ,, ,, + M/600 $MgSO_4$.
C, + 10 per cent tryptic meat broth.
D, ,, ,, ,, +M/600 $MgSO_4$.

TABLE III.—*Comparison of Effects of Broth and Mixed Amino-acids With and Without Mg^{++} on Nitratase Adaptation.*

	Nitratase activity (μ Mols. nitrite/mg./hr.).
Before adaptation	0·23
After 90 min. adaptation :	
+ 18 mixed amino-acids (M/4000 each)	0·98
+ ,, ,, ,, + M/600 Mg^{++}	3·02
+ 10% tryptic meat broth	5·62
+ ,, ,, + M/600 Mg^{++}	6·45

All tubes contained M/5 phosphate, M/100 glucose, M/100 sod. nitrate and 0·2 mg./ml. cells.

Final concentration of added N :

Amino-acids	0·219 mg./ml.
Broth	0·720 ,,

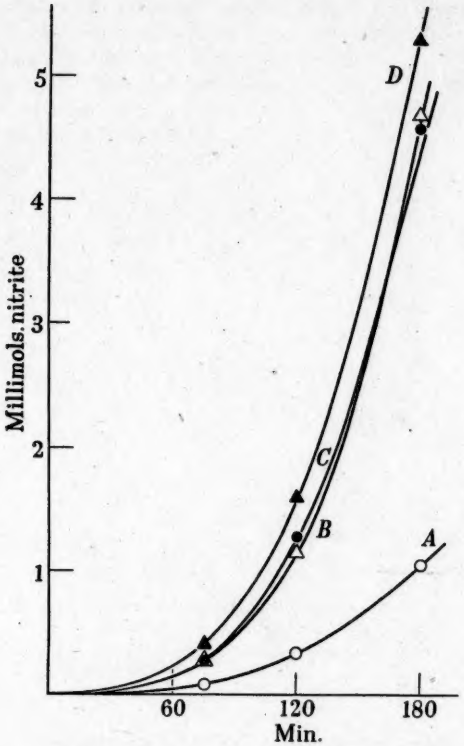

FIG. 4.—Effect of Mg^{++} and buffer concentration on nitratase adaptation.

A, M/5 Phosphate buffer.
B, ,, ,, + M/600 $MgSO_4$.
C, M/10 Phosphate buffer.
D, ,, ,, + M/600 $MgSO_4$.

TABLE IV.—*Effect of Phosphate Concentration and Mg^{++} on Nitratase Adaptation.*

	Nitratase activity (μ Mols. nitrite/mg./hr.).
Before adaptation	0·12
After 3 hours' adaptation:	
M/5 phosphate	1·82
,, ,, + M/600 Mg^{++}	8·08
M/10 phosphate	10·00
,, ,, + M/600 Mg^{++}	9·04

All tubes contained M/100 glucose, M/100 sod. nitrate and 0·2 mg./ml. cells.

to previously (Pollock, 1946). Fig. 5 and Table V show that in the presence of Mg^{++} this " dilution discrepancy " is considerably reduced though not abolished, and suggest that Mg ions may play some part in the phenomenon.

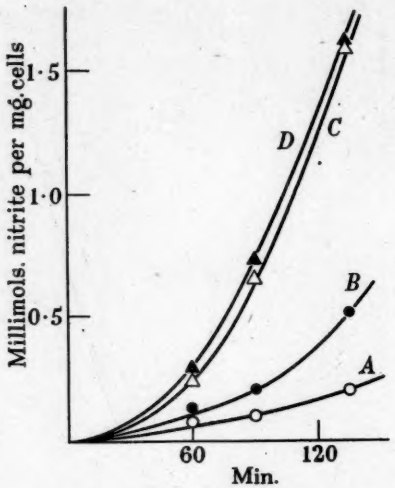

FIG. 5.—Effect of Mg^{++} on rate of nitratase adaptation *per cell* by "dilute" and by " concentrated " cell suspensions.

A, 0·2 mg./ml. cells.
B, " " + M/600 $MgSO_4$.
C, 2·0 mg./ml. cells.
D, " " +M/600 $MgSO_4$.

TABLE V.—*Effect of Concentration of Suspension on Nitratase Adaptation With and Without* Mg^{++}.

	Nitratase activity (μ Mols. nitrite/mg./hr.).
Before adaptation	0·12
After 130 minutes' adaptation :	
0·2 mg./ml. cells	0·76
0·2 " " + M/600 Mg^{++} . .	1·42
2·0 mg./ml. cells	1·54
2·0 " " + M/600 Mg^{++} . .	1·96

All tubes contained M/5 phosphate, M/100 glucose and M/50 sod. nitrate.

Fig. 6 shows that the inhibitory effect of M/5 phosphate could be almost completely reversed by Mg^{++}. It can also be seen that phosphate did not appreciably inhibit adaptation until this concentration of M/5 was reached, but that M/2 was very markedly inhibitory. Moreover, while Mg^{++} almost completely reversed the inhibition of M/5 phosphate, it had little effect on the inhibition by M/2 phosphate, and increasing the Mg^{++} concentration up to M/60 had

no further effect. This suggests that the inhibitory action of the phosphate cannot be ascribed simply to the formation of some insoluble complex with essential Mg inside the cells.

The Mg effect was found to be quite specific. Cd^{++}, Ca^{++}, Fe^{++}, Mn^{++}, Al^{+++} and Zn^{++} at a concentration of M/600 were quite inactive; while Co^{++}, Ni^{++} and Cu^{++} were slightly inhibitory.

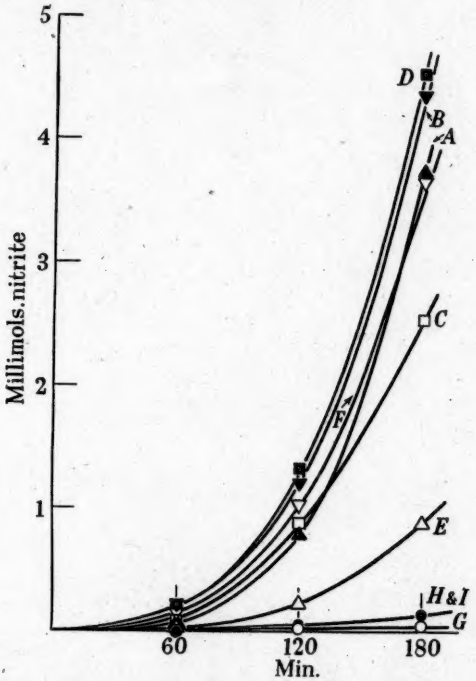

FIG. 6.—Effect of Mg++ and buffer concentration on nitratase adaptation.

A, M/20 Phosphate buffer.
B, ,, ,, + M/600 $MgSO_4$.
C, M/10 Phosphate buffer.
D, ,, ,, + M/600 $MgSO_4$.
E, M/5 Phosphate buffer.
F, ,, ,, + M/600 $MgSO_4$.
G, M/2 Phosphate buffer.
H, ,, ,, + M/600 $MgSO_4$.
I, ,, ,, + M/60 $MgSO_4$.

The effect of Mg^{++}, using similar "dilute" (0·2 mg./ml. final) suspensions, was tested upon the rate of uptake of O_2 with glucose, and upon the rates of nitrate reduction with formate at 35° and with glucose at 45° (that is under conditions where adaptation does not take place), and was found to be negligible (Table VI).

TABLE VI.—*Effect of Mg^{++} on O_2 Uptake and Rate of Nitrate Reduction by Washed Suspensions.*

		Mean rate of O_2 uptake (μ Mols./mg./hr.).
At 35°	Glucose only	4·25
	,, + M/600 Mg^{++}	4·22

All cups contained M/5 phosphate and 0·2 mg./ml. cells (final vol. 2·5 ml.).
Duration of experiment: 160 minutes.

		Nitratase activity (μ Mols. nitrite/mg./hr.).
At 45°	Glucose only	1·68
	,, + M/600 Mg^{++}	1·68
At 35°	Formate only	3·71
	,, + M/600 Mg^{++}	3·68

All tubes contained M/100 sod. nitrate, M/5 phosphate and 0·2 mg./ml. cells.
Duration of experiments: 60 minutes.

A specific stimulatory effect of Mg^{++} similar to that shown with nitratase adaptation could be demonstrated on growth of " 1433 " in a synthetic medium of the following composition: KH_2PO_4, 0·45 per cent; NH_4Cl, 0·05 per cent; $(NH_4)_2SO_4$, 0·057 per cent; $FeSO_4$, 0·002 per cent; glucose, M/200. Table VII shows that Mn^{++} had no effect in replacing Mg^{++} for promoting growth.

TABLE VII.—*Effect of Mg^{++} and Mn^{++} on Growth in a Glucose-NH_3 Medium.*

Medium.	Growth. 24 hr.	48 hr.
Basal*	—	tr.+
,, + M/6000 Mg^{++}†	tr.	+++±
,, + M/6000 Mn^{++}†	—	tr.

Inoculum: Approximately 20 cells.
Incubation: In 50 ml. flask in air + 5% CO_2 at 35°.
* See text.
† Added as sulphate.

Nitratase adaptation and cell growth.

In quantitative studies of cell growth it is preferable to be able to follow growth by some method which measures the increase in total cell protoplasm. Obviously the estimation of bacterial dry weights would be the most accurate; but, apart from some intrinsic disadvantages, it cannot be applied practicably to the early stages of growth in relatively small volumes of culture medium. Total or viable counts are liable to be misleading, since they take no account of changes in cell size that are known to occur, especially during the early phases of the growth cycle. The method used here, that of direct opacity measurements in a sensitive and accurate absorptiometer, has been employed successfully on many occasions (Monod, 1942), and is probably the most accurate method

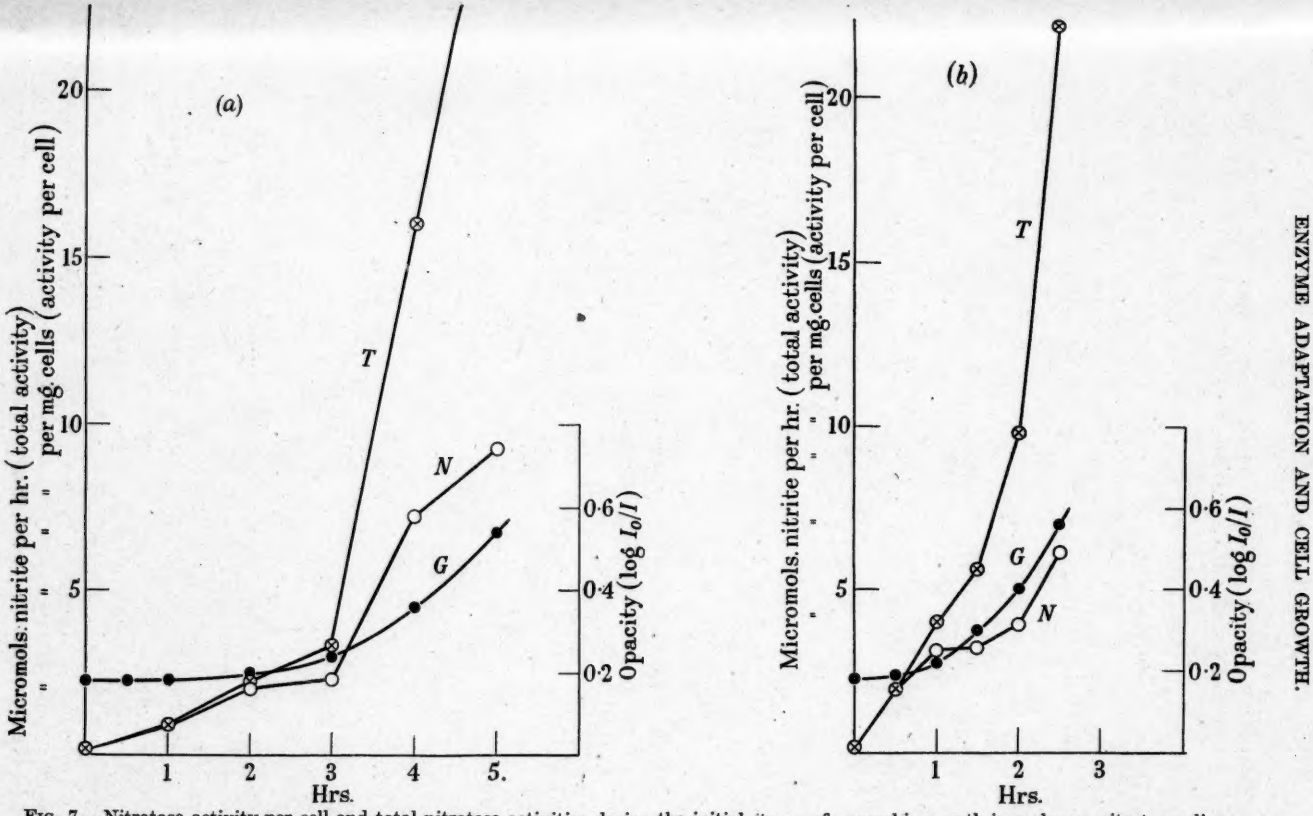

FIG. 7.—Nitratase activity per cell and total nitratase activities during the initial stages of anaerobic growth in a glucose-nitrate medium.

G, Opacity of culture (log Io/I).
N, Nitratase (μ mols. nitrite formed/mg. dry weight/hr.).
T, Total nitratase (μ mols. nitrite formed/hr.).

(*a*) With M/100 NH_4Cl.
(*b*) With M/4000 each of 18 amino-acids.

that is convenient. It has been shown that opacity measurements are a far nearer approximation to actual bacterial mass than total or viable counts (Loofbourow and Dwyer, 1938), and experiments performed by us showed on the whole good agreement between the two. The main disadvantages are: (*a*) in order to follow the early stages of growth it is necessary to use an inoculum that in ordinary growth experiments would be regarded as very large (e.g. about $2{\cdot}5 \times 10^8$/ml.); and (*b*) changes in cell volume due merely to the intake or output of water will be estimated as changes in cell mass. It is important, therefore, to bear these points in mind when employing this otherwise satisfactory and simple method of following bacterial growth.

Fig. 7 (*a*) and (*b*) shows the increase in nitratase activity in relation to the early growth stages with NH_4Cl and amino-acids respectively as N source. . It is clear that the increase of nitratase precedes and greatly exceeds the growth of the cell as a whole. For instance, in the experiment illustrated in Fig. 7 (*a*) the

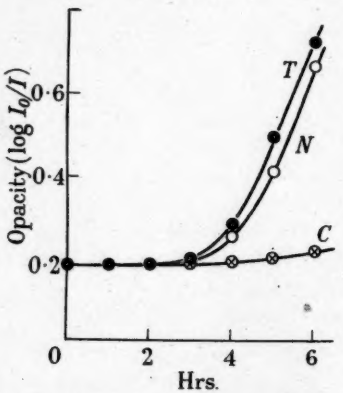

FIG. 8.—Initial growth (anaërobic, at 35°) in a glucose-NH_3 medium.
C, No H-acceptor.
N, + M/100 $NaNO_3$.
T, + M/100 $Na_2S_4O_6$.

initial nitratase activity was 0·15, rising to 0·92 after 60 minutes (accompanied by no increase in opacity), and to 2·32 after 120 minutes (with only a 10 per cent increase in opacity): that is a 15-fold increase in activity associated with only a 10 per cent increase in mass. In other words, nitratase adaptation occurs to a very great extent during what is commonly known as the " lag phase."

The question immediately arises as to whether this enzymatic adaptation might in any way directly influence the rate of cell growth. Under conditions where the rate of nitrate reduction might be expected to be a limiting factor for the supply of energy for cell growth as a whole, it appeared likely that the initial speed of growth of the cells in an inoculum would be proportional to their nitratase activity. Quastel, Stephenson and Whetham (1925) showed that anaërobic growth of *Bact. coli* with lactate as sole source of carbon would only occur in the presence of a reducible H-acceptor such as nitrate. Fig. 8 shows the effect of tetrathionate and nitrate on the growth of " 1433 " with glucose, in the absence

of O_2. Glucose can be metabolized anaërobically, and growth will occur (as with *Bact. coli*), though slowly, in the absence of a H-acceptor; but the effect of adding tetrathionate or nitrate is striking, and their efficiency in promoting growth should depend on the rate at which they are reduced.

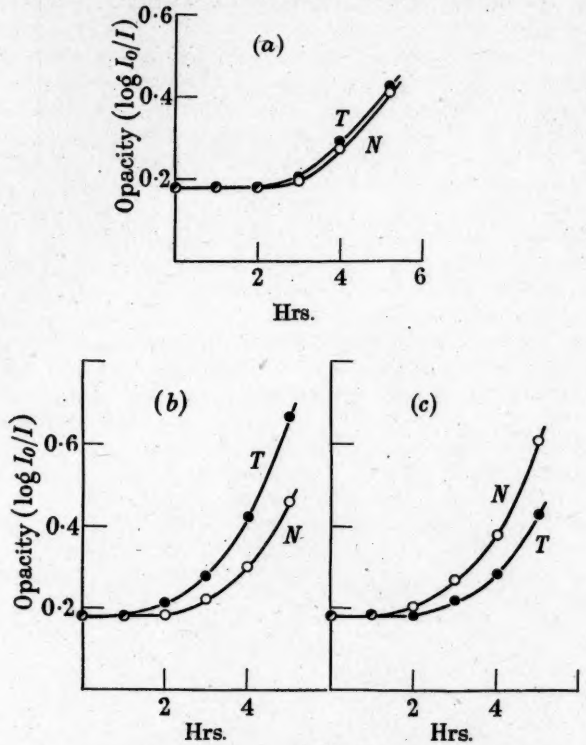

FIG. 9.—Comparative rates of growth (anaërobic at 35°) in a glucose-NH_3 medium, with nitrate (N) or tetrathionate (T) as H-acceptor.

(*a*) Control (unadapted) cells.
(*b*) Tetrathionate-adapted cells.
(*c*) Nitrate-adapted cells.

In order to test the effect of previous adaptation upon the rates of growth in tetrathionate and nitrate, 1 ml. lots from the same cell suspension were treated at 35° as follows:

Designation.	Water.	M/1 phosphate buffer.	M/10 mannitol.	M/10 sod. nitrate.	M/10 sod. tetra-thionate.
Control ("C" cells)	3·5	1·5	1·5	—	—
Nitrate-adapted ("N" cells)	2	1·5	1·5	1·5	—
Tetrathionate-adapted ("T" cells)	2	1·5	1·5	—	1·5

This adaptation treatment was allowed to proceed until all the tetrathionate was reduced (about 3½ hours), and the cells were centrifuged down and washed twice with water, resuspended, and a sample of each inoculated into both the tetrathionate and the nitrate growth media, the opacities being recorded every hour (Fig. 9). Table VIII gives the nitratase and tetrathionase activities of the

TABLE VIII.—*Nitratase and Tetrathionase Activities of Differently Adapted Suspensions Used in Experiment Illustrated in Fig.* 9.

Treatment.	Nitratase.*	Tetrathionase.*
Glucose only (" C " cells) . . .	0·19	0·27
,, + nitrate (" N " cells) . .	3·55	0·27
,, + tetrathionate (" T " cells) .	0·19	6·65

* Expressed as μ mols. nitrite or thiosulphate formed per mg. dry weight of cells per hour.

three differently treated suspensions. Fig. 9 shows that the control unadapted cells grew equally well in tetrathionate and nitrate; nitrate adapted cells grew relatively better in the nitrate medium, while tetrathionate adapted cells grew better in the tetrathionate medium. The differences are not great, but were found quite consistently in repeated experiments. The significant fact is that they were specific.

DISCUSSION.

As emphasized previously, adaptation of the nitratase system has been studied functionally, and no attempt has been made to determine the exact nature of the processes involved. The simplest, and in many ways the most attractive, hypothesis is that adaptation depends upon the synthesis or remoulding of enzyme protein itself. The stimulating effect of an external supply of N on galactozymase adaptation has been reported by Spiegelman and Dunn (1947) for yeast cells; and the marked accelerating effect of amino-acids upon bacterial nitratase adaptation, the extent of which was found to depend upon the number of different acids added, is consistent with the possibility that the relevant factor is synthesis of new protein. The fact that adaptation will occur in the absence of assimilable N is no evidence against this hypothesis, since there would almost certainly be an internal supply of metabolizable N sufficient to allow the formation of the small amount of protein necessary to account for a 10- or 20-fold increase in the activity of a single enzyme.

The pronounced specific stimulating effect of Mg^{++} on nitratase adaptation shows that it must be concerned at some point in the chain of reactions which constitute the phenomenon, but the fact that it is only clearly manifested in the presence of a high concentration (M/5) of phosphate and when a relatively thin suspension of cells is being employed emphasizes the complexity of the problem. Mg^{++} had no effect on O_2 uptake with glucose or upon the rate of action of nitratase itself. These facts show that the action of Mg^{++} is upon adaptation itself, and does not appear to be involved in the associated processes of glucose metabolism. The fact that no marked Mg^{++} stimulation occurred in low concentrations of phosphate suggests that the enzyme system or systems responsive to Mg^{++} only became limiting when inhibited by the higher concentrations of phosphate. Mg^{++} is well known to activate many different bacterial

enzyme systems, but, so far as it has been tested, it can be replaced (with two exceptions) by Mn^{++} or other metals. The exceptions are the glycerophosphatase and hexose diphosphatase of *Cl. acetobutylicum* and *Prop. jensenii* described by Pett and Wynne (1933), both of which were specifically stimulated by Mg^{++}. It is also interesting to note that the glycerophosphatase is recorded as being 80 per cent inhibited by M/100 phosphate, so that this enzyme system has two points of similarity to nitratase adaptation. It is, of course, known that Mg^{++} is specifically required for the growth of a number of species of micro-organisms, and certainly cannot be replaced by Mn^{++} for the growth of " 1433," but this is presumably only a restatement of the problem and throws no further light on its mode of action.

Clearly, it is not possible to interpret the relatively slight effect of Mg^{++} with concentrated cell suspensions as being due to there being enough Mg^{++} present in the preparation already. The effect of phosphate concentration referred to previously shows that it might equally well be due to some unknown substance liberated during metabolism raising the rate of adaptation up to a level at which another reaction, insensitive to Mg^{++}, became the limiting factor. It can, however, be accepted that the dilution effect is not due to a simple diffusion of Mg^{++} from within the cell, since repeated washing of the cells with water or prolonged dialysis did not significantly affect the more rapid rate of adaptation attained by concentrated suspensions.

In view of the identification of Mg^{++} in the washings from Ford filter pads, it is of interest to note that the presence of " bacteriological growth substances " in Seitz and Ford filter pads has been reported previously by Hunter (1943), who identified Ca^{++} and Fe^{+++}, but made no mention of Mg^{++}.

The experiments here reported on the rise of nitratase activity during the lag and early growth phases of " 1433 " in a nitrate medium, and the effect of previous adaptation treatment on subsequent growth in the presence of the specific substrate as H-acceptor, show that specific adaptation must occur before, and as a preliminary to anaërobic growth in nitrate or tetrathionate. From a purely teleological point of view it is clearly to the advantage of cells incubated under such conditions to be able to reduce nitrate (or tetrathionate)—the only available H-acceptor—as rapidly as possible; and it is only logical to conclude—as indeed appears to be the case—that the greater the previous adaptation, the sooner and the more rapidly will growth occur. Until recently, it was widely held that enzyme adaptation could not occur in the absence of growth. In the cases of nitratase and tetrathionase, however, it is more reasonable to conclude that growth cannot occur in the absence of adaptation. It is not improbable that the same principle applies to other enzymes, and may contribute to an explanation of the so-called " lag phase " of bacterial growth—most adequately defined as the period before maximal rate of growth.

Some doubts have on occasions been thrown on the reality of the lag period (Hershey, 1938, 1939) and it is admittedly true that under appropriately chosen conditions the lag can be completely abolished (Chesney, 1916). The increase in metabolic activity per cell (O_2 uptake—Martin, 1932, and Walker, Winslow, Huntingdon and Mooney, 1934; CO_2 production—Walker *et al.*, 1934; heat production—Schmidt and Bayne-Jones, 1933) that is known to occur during the lag phase can in part be explained by the known fact that

during this period bacteria increase in size before cell division begins. Nevertheless, as pointed out by Henrici (1928), there appears in some cases to be an absolute phase of true lag even before cells begin to increase in size; and even if enzyme activities are expressed per unit of cell size they have been shown in some instances to increase during the lag (Huntingdon and Winslow, 1937). This is also presumably true of better defined enzyme systems such as formic hydrogenlyase of *Bact. coli* (Stephenson and Stickland, 1933), where adaptation occurred in tryptic meat broth before cell multiplication began, and arginine dihydrolase of streptococci (Gale, 1945), where maximum activity was found at the earliest stage of growth at which it was measured (3 hours' incubation). As interpreted by Hinshelwood (1944), the lag period consists of the time necessary for the accumulation of intermediary substrates and the production of enzymes up to the level necessary for maximal metabolic activity with respect to growth in any one particular medium. Unless such intermediaries and enzymes are already present at an optimal level in the cells of the inoculum (as may happen if they are derived from a culture in the logarithmic phase) some period of lag must inevitably occur.

Two further pieces of work in relation to enzyme adaptation or formation during bacterial lag are worth referring to. Caspersson (1946) and Malmgren and Hedén (1947) report a big increase in the cell nucleic acid content of *Bact. coli* during the lag phase, which Caspersson claims to be an indication of increased rate of protein synthesis. Secondly, Lodge and Hinshelwood (1939) have reported that Mg^{++} has a specific effect in shortening the lag of *Bact. lactis aerogenes*, and that, below the optimal concentration, length of lag is inversely proportional to Mg^{++} concentration. If bacterial lag is largely due to the time necessary for the production of enzymes required for maximal rate of growth, it is not surprising to find independent evidence that (*a*) protein is synthesized during this period; and (*b*) Mg^{++}, already shown to stimulate nitratase adaptation, has a pronounced effect in shortening the lag.

The hypothesis of Monod (1942) that adaptation to certain carbohydrates is a necessary preliminary to growth upon these substances as sole source of carbon is supported by good, though indirect, evidence. But in his experiments, selection of certain cells able to oxidize the specific carbohydrate, although shown to be very unlikely, cannot be completely ruled out. Results with tetrathionase and nitratase, however, where adaptation has been achieved under conditions where growth is known not to occur, show that it contributes to a shortening of the lag for cells growing anaërobically on the specific substrate, and thus confirm Monod's original theory. It does not seem unreasonable to conclude that similar processes of adaptation occur with other enzyme systems during the lag phase, and may prove to be characteristic of the early prelogarithmic growth phases of most micro-organisms.

SUMMARY.

1. Addition of amino-acids greatly increases the rate of nitratase adaptation in washed suspensions of the coliform organism "1433." This stimulation is independent, within wide limits, of the amino-acid concentration, but proportional to the number added.

2. Mg^{++} specifically stimulates nitratase adaptation in "1433."

3. Nitratase adaptation, in the presence of assimilable N, occurs during the lag phase before the commencement of, and, later, very much more rapidly than generalized cell growth.

4. Specific adaptation of cells to tetrathionate or nitrate in the absence of assimilable N confers a specific advantage in subsequent anaërobic growth in tetrathionate or nitrate respectively.

5. The significance of enzyme adaptation in relation to the lag phase of bacterial growth is discussed.

We are much indebted to Sir Paul Fildes, F.R.S., for his advice and interest; also to Miss A. H. Hearle for technical assistance.

Appendix (i).

Amino-acids used.

glycine	(S)	dl-valine	(S)
dl-alanine	(S)	dl-cystine	(S)
dl-tryptophan	(S)	dl-methionine	(S)
dl-tyrosine	(S)	dl-phenylalanine	(S)
dl-glutamic acid	(S)	dl-threonine	(S)
dl-lysine	(S)	dl-isoleucine	(S)
dl-leucine	(S)	l-histidine	
dl-aspartic acid	(S)	l-proline	
dl-serine	(S)	l(+)-arginine	

(S) = synthetic. The concentrations in the text refer to those of the l-isomer only.

Appendix (ii).

Growth Factors Tested.

	Conc.		Conc.
Biotin	10·0 mμg./ml.	Inositol	9·0 μg./ml.
Nicotinamide	1·0 μg./ml.	Thymine	6·5 ,,
Ca pantothenate	0·75 ,,	Uracil	5·5 ,,
Pyridoxin	2·5 ,,	Adenine sulphate	10·0 ,,
Riboflavin	1·2 ,,	Cytosine	5·5 ,,
Thiamin HCl	0·5 ,,	Pimelic acid	0·8 ,,
p-amino-benzoic acid	0·45 ,,	Folic acid	1·0 ,,
Guanine sulphate	11·0 ,,	Haemin	2·0 ,,

REFERENCES.

Caspersson, T.—(1946) *Symp. Soc. exp. Biol.*, **1**, 144.
Chesney, A. M.—(1916) *J. exp. Med.*, **24**, 387.
Gale, E. F.—(1945) *Brit. J. exp. Path.*, **26**, 255.
Henrici, A. T.—(1928) 'Morphological Variation and Rate of Growth of Bacteria.' London (Baillière, Tindall & Cox), p. 69.
Hershey, A. D.—(1938) *Proc. Soc. exp. Biol. Med., N.Y.*, **38**, 127.—(1939) *J. Bact.*, **37**, 285.
Hinshelwood, C. N.—(1944) *Biol. Rev.*, **19**, 135.—(1946) 'Chemical Kinetics of the Bacterial Cell.' Oxford (Clarendon Press).
Hunter, G. J. E.—(1943) *Biochem. J.*, **37**, 577.

HUNTINGDON, E., AND WINSLOW, C. E. A.—(1937) *J. Bact.*, **33**, 123.
KNOX, R., AND POLLOCK, M. R.—(1944) *Biochem. J.*, **38**, 299.
LODGE, R. M., AND HINSHELWOOD, C. N.—(1939) *J. chem. Soc.*, 1692.
LOOFBOUROW, J. R., AND DWYER, SISTER C. M.—(1938) *Studies Inst. Div. Thom.*, **2**, 129.
MALMGREN, B., AND HEDÉN, C. G.—(1947) *Nature*, **159**, 577.
MARTIN, O. S.—(1932) *J. gen. Physiol.*, **15**, 691.
MONOD, J.—(1942) 'Recherches sur la Croissance des Cultures Bactériennes.' Paris (Hermann).
PETT, L. B., AND WYNNE, A. M.—(1933) *Biochem. J.*, **27**, 1660.
POLLOCK, M. R.—(1945) *Brit. J. exp. Path.*, **26**, 410.—(1946) *Ibid.*, **27**, 419.
Idem AND KNOX, R.—(1943) *Biochem. J.*, **37**, 476.
QUASTEL, J. H., STEPHENSON, M., AND WHETHAM, M.D.—(1925) *Ibid.*, **19**, 305.
SCHMIDT, C. F., Jnr., AND BAYNE-JONES, S.—(1933) *J. cell. comp. Physiol.*, **3**, 405.
SPIEGELMAN, S., AND DUNN, R.—(1947) *J. gen. Physiol.*, **31**, 153.
Idem, REINER, J. M., AND MORGAN, I.—(1947) *Arch. Biochem.*, **13**, 113.
STEPHENSON, M., AND STICKLAND, L. H.—(1933) *Biochem. J.*, **27**, 1528.
TINSLEY, J., AND PIZER, N. H.—(1945) *J. Soc. chem. Ind., Lond.*, **64**, 182.
WALKER, H. H., WINSLOW, C. E. A., HUNTINGDON, E., AND MOONEY, M. G.—(1934) *J. Bact.*, **27**, 303.

"TRAINING" OR MUTATION OF BACTERIA.

PAUL FILDES AND K. WHITAKER.

From the Unit for Bacterial Chemistry (Medical Research Council) at the Lister Institute, London, S.W. 1.

Received for publication May 4, 1948.

FOR some years reference has been made to the "training" of exacting bacteria to grow on media deficient in some essential metabolite. For instance, Fildes, Gladstone and Knight (1933) described the training of *Bact. typhosum* to grow in media devoid of tryptophan, while Fildes (1945) referred to a strain of *Bact. typhosum* which had been trained to dispense with indole. During this time little attention had been given to the mechanism of the training process, but it was certainly supposed that the enzyme make-up of the bacteria became altered as a result of a stimulus produced by deficiency of the metabolite. On the other hand, during recent years the teaching of geneticists has taken so firm a hold on bacteriology that it has become possible to describe bacteriologists, who have not conformed, as defenders of "one of the last strongholds of Lamarkism" (Luria, 1947). The subject is, therefore, reconsidered from the genetic point of view, accepting the existence of genes in bacteria from analogy with sexually reproducing organisms susceptible to normal genetical techniques of study.

The change in enzymes observed in training would be considered to be a sequel of some genetic upset (mutation) occurring in a random manner, suggesting pure chance and unrelated to the environment. In the tryptophan example it would be a question of showing that normal cultures of *Bact. typhosum* always contain mutants which grow without tryptophan if the number of divisions of

the bacteria in the culture has been sufficiently large to ensure the probability. Further, the concentration of tryptophan should have no influence on the occurrence of mutants.

Most nutritional mutations of bacteria which have been recorded have been losses of enzymes due to destruction of the controlling gene. In the present instance it is a question of some disturbance of gene distribution or function which results in the gain of an enzyme. Mutations resulting in a greater synthetic power have been recorded by Monod (1946) and Wollman (1947). The former referred to a strain of *Bact. coli* which required methionine for normal growth, but produced mutants which grew equally without methionine. The latter described a strain of *Bact. coli* which he had selected by phage resistance, and which was found to require tryptophan for growth. This strain produced mutants, at the rate of 1 to 10^5–10^8 normals, which grew without tryptophan.

It will be noticed that the " normals " which produced the " mutants " in these cases were themselves mutants from the normal. It must be assumed, therefore, that these mutations are reversible. In the present work the normals are typhoid bacilli as usually met with in medicine, i.e. devoid of the enzyme which uses anthranilic acid in the synthesis of indole. In the mutant this enzyme has been recovered.

EXPERIMENTAL.

Strains.—The strain of *Bact. typhosum* (NCTC 3390) used in this work has been studied for some years in this laboratory, and found to be incapable of growing without indole or tryptophan. It is referred to as " N " (normal). Since it is liable to change in the direction of acquiring the power to grow without tryptophan, it is maintained in the dry state. " M " (mutant) refers to a strain derived from N by " training." This can grow without indole or tryptophan.

Culture media.—" Casein agar " or "broth " refers to media consisting of 0·5 per cent " vitamin-free " hydrolysed casein (Ashe Laboratories, Ltd., 120, Victoria Street, London S.W. 1) with M/30 phosphate buffer, M/30,000 cystine, M/6000 Mg and M/20 lactate. These are tryptophan-free media to which *dl*-tryptophan (synthetic) can be added as required.

Casein agar plates were incubated in air + 5 per cent CO_2. Casein broth was usually made up in 250 or 500 c.c. amounts in 1½ l. Erlenmeyer flasks and agitated in the hot room.

Behaviour of N and M in culture media.

Casein broth.—There was no demonstrable difference between the growth of N on casein broth + excess tryptophan (10^{-4} M) and M on the same medium with or without tryptophan. Both strains starting with an inoculum of 5–10 bacteria/c.c., after a lag of 1 to 3 hours, entered the log phase which ended at a concentration of about 10^8 bacteria/c.c. The generation time was 45 minutes and the peak of growth usually attained 5×10^9 bacteria/c.c. or somewhat higher. Plots of the growth of the two strains in the same culture were practically superimposed. The effect of reducing the concentration of tryptophan in cultures of N was to reduce the peak of growth to a roughly proportional extent. 10^{-5} M was slightly limiting, the peak attaining $1–5 \times 10^9$ bacteria/c.c. At 10^{-6} M the peak was approximately 10^8 bacteria/c.c., and at 10^{-7} M, 10^7 bacteria/c.c. Over this range there was no difference in the generation times. At 10^{-8} M the mass of

growth fell disproportionally to about 10^5 bacteria/c.c., while the generation time was prolonged 4 times. When no tryptophan was added, viable counts at zero time and subsequently remained unchanged, indicating that there was no demonstrable tryptophan in the casein hydrolysate.

Casein agar.—There was also no demonstrable difference between the growth of N or M on this medium when excess tryptophan was present or between the growth of M in the absence of tryptophan.

In 24 hours colonies measured 1·3–1·5 mm., in 48 hours 2·5–3·0 mm., with a continual increase on longer incubation.

When it was necessary to make heavy inoculations of N on casein agar without tryptophan, it was found that growth took place to an extent invisible to the eye, but seen under the microscope to consist of a fine film or crowded irregular transparent colonies. On further incubation these fine colonies did not increase in size, and on subculture to the same tryptophan-free medium did not grow. It was obvious that the abortive growth was due to a carry-over of tryptophan necessarily present in the previous culture. However, the effect was not much reduced by washing the inoculum once, and it is supposed that the bacteria themselves have concentrated tryptophan so much intracellularly as to supply their wants for a few generations after transfer to a tryptophan-free medium.

Viable counts and mutants.

Viable counts which do not differentiate normals from mutants were carried out on nutrient agar or casein agar + tryptophan. Measured quantities of dilutions suitable to give counts of at least 500 were spread on the media and counted in 24 hours.

The estimation of mutants with sufficient accuracy for quoting mutation rates is probably impossible. It was soon found that cultures of N did, in fact, contain units which grew without tryptophan, and thus must be called mutants, but in such small numbers that they could not be detected without plating out with them enormous numbers of N. The normal procedure was to plate out 0·1 c.c. of a specimen on each of ten plates of casein agar without tryptophan. This might entail implanting 10^8 N or more per plate.

Under these circumstances, in 24 hours N grew, as already mentioned, as a fine film. Any mutants which developed also grew at the same rate as a known mutant deliberately spotted on the plate as a control. If the 24-hour colonies were marked and observation continued for two or three or more days, it was found that the number of mutant colonies continually increased. These have not been taken into account because it is impossible to decide whether they were present as mutants in the specimen under examination, or had developed as mutants from the film growth of N on the agar plates under precisely the same influences as may have caused them in the specimens. It is more probable that these later colonies are products of the plates rather than of the specimens, because of their timing, and because they do not occur when the number of mutants is large enough to allow high dilution of the specimen.

Incubation technique.

For convenience in making counts at short intervals over a prolonged time, the cultures were removed from the hot room at night and plunged into iced

water. Next day they were rapidly warmed and continued at 37° C. This technique had no demonstrable influence on growth even in the log phase. The times recorded are, therefore, those at which the cultures were maintained at 37° C.

MUTANTS IN NUTRIENT AGAR CULTURES OF *Bact. typhosum.*

The normal nutrient agar cultures of typhoid 3390 which we had found to be incapable of growing without tryptophan, were examined for mutants which could grow without tryptophan.

Four petri plates of nutrient agar were inoculated with N, grown for 20 hours and the culture scraped off into 20 c.c. water, centrifuged, and the deposit made up to $5{\cdot}2 \times 10^{10}$ bacteria/c.c. with the aid of Hilger's Spekker absorptiometer. 0·1 c.c. of this suspension and 1/10 dilutions thereof were distributed on plates of casein agar and into flasks of casein broth, in both cases without tryptophan.

The results of incubation for 1 and 3 days are shown in Table I. It is seen that 1130 M colonies developed from an implantation of $5{\cdot}2 \times 10^9$ N or roughly 1 M colony for every 5×10^6 N implanted. The liquid cultures are in agreement, since growth occurred from $5{\cdot}2 \times 10^6$, but not from $5{\cdot}2 \times 10^5$. Twelve colonies taken at random were suspended in water, and approximately 10^4 organisms from each planted in casein broth. Similar numbers of organisms were taken from the liquid cultures and transferred to the same medium. These all grew normally like "trained" strains. Further M/10,000 tryptophan added to the ungrown liquid cultures produced normal growth.

Between the colonies on the plates were large numbers of N bacteria which could only grow abortively owing to absence of tryptophan. These were sub-cultured to nutrient agar, thick suspensions made up and inoculated again on casein agar without tryptophan. This was, in effect, a deliberate selection of N strains. This process was repeated five times in series and M units persisted thus:

	1 in:
Original experiment . . .	5×10^6 normals implanted.
1st repeat in series . . .	2×10^6 ,, ,,
2nd ,, ,, . . .	5×10^6 ,, ,,
3rd ,, ,, . . .	$2{\cdot}4 \times 10^7$,, ,,
4th ,, ,, . . .	5×10^7 ,, ,,
5th ,, ,, . . .	$6{\cdot}5 \times 10^6$,, ,,

TABLE I.

Number of organisms implanted. $5{\cdot}2 \times$	Number of colonies on agar. 1 day.	Growth in liquid. 1 day.	Growth in liquid. 3 days.
10^9	1130	+++	+++
10^8	77	+	+++
10^7	1	tr.	+++
10^6	0	0	+++
10^5	0	0	0
10^4	0	0	0
10^3	0	0	0

Plus signs are roughly in proportion to the opacity; tr. = trace of visible growth.

On other occasions suspensions from young agar cultures have been tested and found to contain 1 M/8 $\times$ 10^7, 6 $\times$ 10^7, 3·4 $\times$ 10^8 and 5 $\times$ 10^8 N.

In view of these results it cannot be doubted that cultures of N on routine laboratory media normally contain units which can grow without tryptophan. The proportion of these units to normals is very small, varying between 1 in 2 $\times$ 10^6 and 5 $\times$ 10^8.

These quantitative results explain the observation that in training experiments a small inoculum of N will not grow in the absence of tryptophan, whereas a large will do so. Our " small " inoculum consisted of 10^4 bacteria in 10 c.c. ≡

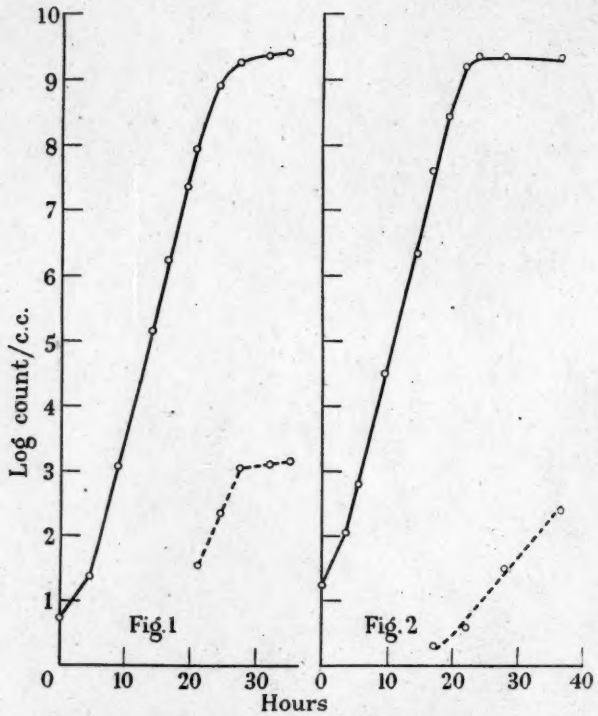

FIG. 1.—Result of growing N on casein broth containing 10^{-4} M tryptophan.
FIG. 2.—Result of growing N on casein broth containing 10^{-5} M tryptophan.
——— = total count ; - - - - - - = mutants.

10^3/c.c. The chance of including a mutant in this number is small, and thus no growth occurred. The larger the inoculum, however, the more likely is a mutant to be included.

THE DEPENDENCE ON TRYPTOPHAN.

The conditions in an agar culture are always less uniform than in a liquid, and it may be argued that in every agar culture the bacteria in the centre of a

colony are deficient in nutrients compared with those on the periphery. Thus, some bacteria in every agar culture may be starved of tryptophan and so become " trained " to synthesize it.

Experiments were therefore carried out in casein broth. The procedure was as follows:

The standard culture was 250 c.c. casein broth in a 1½ l. Erlenmeyer flask, continually shaken in the hot room. The inoculum was about 10 N/c.c. in the culture, measured by dilution of a suspension of a nutrient agar culture of N

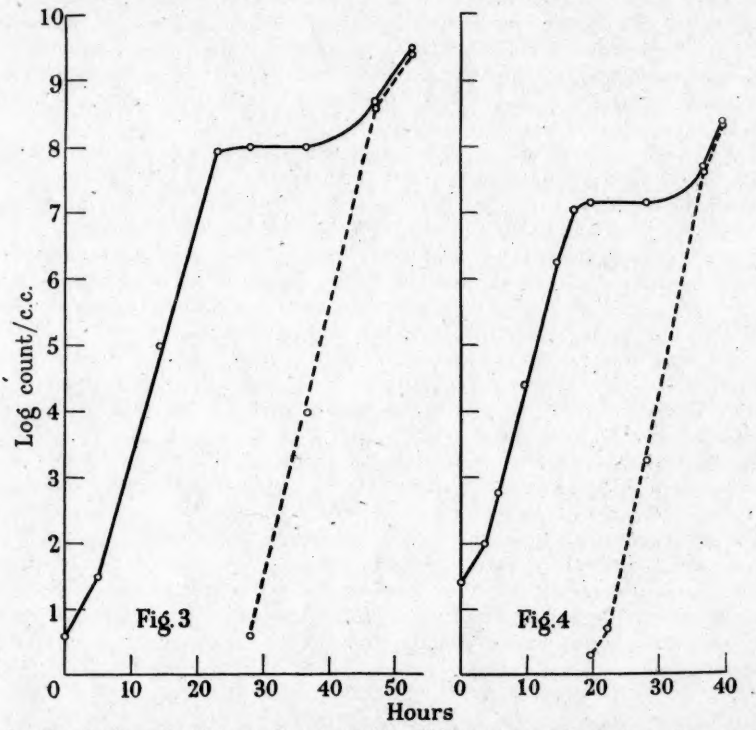

FIG. 3.—Result of growing N in a culture limited to 10^8 bacteria/c.c. by reduction of tryptophan.
FIG. 4.—Result of growing N in a culture limited to approximately 10^7 bacteria/c.c. by reduction of tryptophan.
——— = total count; - - - - - - = mutants.

estimated by Hilger's Spekker absorptiometer. *dl*(synthetic)-tryptophan was added to make known concentrations indicated in terms of the active isomer. Viable counts were made from time to time on nutrient agar giving the sum of the total N + M. Mutant counts were made on casein agar without tryptophan.

Examples of results are given in Fig. 1, 2, 3 and 4.

Fig. 1 shows the result of growing N on casein broth containing 10^{-4} M tryptophan, which is excess for producing full growth on this medium. The culture is limited by lack of some other nutrient constituent than tryptophan. It will be

noticed that mutants were observed after 21 hours' incubation when the viable count was $8{\cdot}9 \times 10^7$ /c.c. These mutants underwent about four generations before growth ceased, and during the same period the parent strain passed through about five.

Fig. 2 shows the result when the culture is slightly limited by a reduced concentration of tryptophan (10^{-5} M). Mutants were observed after 17 hours' incubation when the viable count was $3{\cdot}1 \times 10^7$/c.c. These mutants were still increasing after seven generations when observation ceased. The parent culture ceased after about five generations.

Fig. 3 shows a culture limited to a peak of 10^8 bacteria/c.c. by reduction of tryptophan. Mutants were observed at 28 hours with a viable count at 10^8/c.c. In this case the mutants behaved quite differently from those shown in Fig. 1 and 2. Continuous growth took place at the normal rate until the main viable count was overtaken and carried up to the limit.

Fig. 4 shows a very similar effect when the culture was limited to approximately 10^7 bacteria/c.c. by reduction of tryptophan.

A culture made with further reduction of tryptophan which limited growth to 2×10^5 bacteria/c.c. did not produce mutants during 100 hours.

It is concluded from these and other experiments that mutants normally appear in cultures which attain over 10^7 bacteria per c.c., or sometimes at a lower growth level. Since the level of growth depends upon the tryptophan concentration, it follows that mutation normally occurs with concentrations of tryptophan 10^{-7} M or greater, but seldom with lower concentrations. This is merely a reflection of the view that mutation occurs very rarely in terms of the number of bacterial divisions. It is not in accordance with a view that deprivation of tryptophan induces mutation.

The degree to which a mutant grows in the mother culture depends entirely upon the state of exhaustion of the mother culture at the time of appearance of the mutation. In excess of tryptophan a mutant can only grow for the same number of generations as can the mother cell before the nutrients are exhausted. When, however, growth of the mother cells is limited by deficiency in tryptophan, then the mutants can continue to grow in excess of the mother cells to exhaustion of the medium. A partial deprivation of tryptophan by creating conditions selective for the mutant thus favours its detection, although it does not effect its occurrence.

Fig. 5 illustrates the argument diagrammatically.

There are references in the literature to failure of continued growth of mutants in the original culture, and this has been ascribed to inhibition by toxic products of the growth of the mother cells (Zamenhof, 1946). In the particular mutation discussed here it is clearly unnecessary to adopt this explanation, and, in fact, the mother culture filtered and reconstituted with nutrients was not inhibitory to growth of mutants. It is also unnecessary to assume some abnormality in the growth characters of a newly developed mutant as compared with an "established" mutant, because an established mutant added to cultures behaves in precisely the same way.

In the literature dealing with training and similar effects, the impression is given that training becomes established after a considerable lapse of time during which there is no growth. In the present experiments mutation has always occurred at or before the peak of growth of the mother cell, but, of course, the

sequel to the mutation has not been visible to the eye until some hours later. Taking the early paper of Fildes, Gladstone and Knight (1933) (Table V), it is seen that trained growth occurred in a tube between four and ten days after inoculation. This tube contained rather less than 10^{-6} M tryptophan, which is enough to allow growth of 10^8–10^7 N/c.c. This, being a concentration hardly visible in a test-tube to the eye, would be recorded as " no growth." Further, the culture medium and conditions, apart from tryptophan, were clearly very suboptimal. It may be suspected that the culture might easily take four days

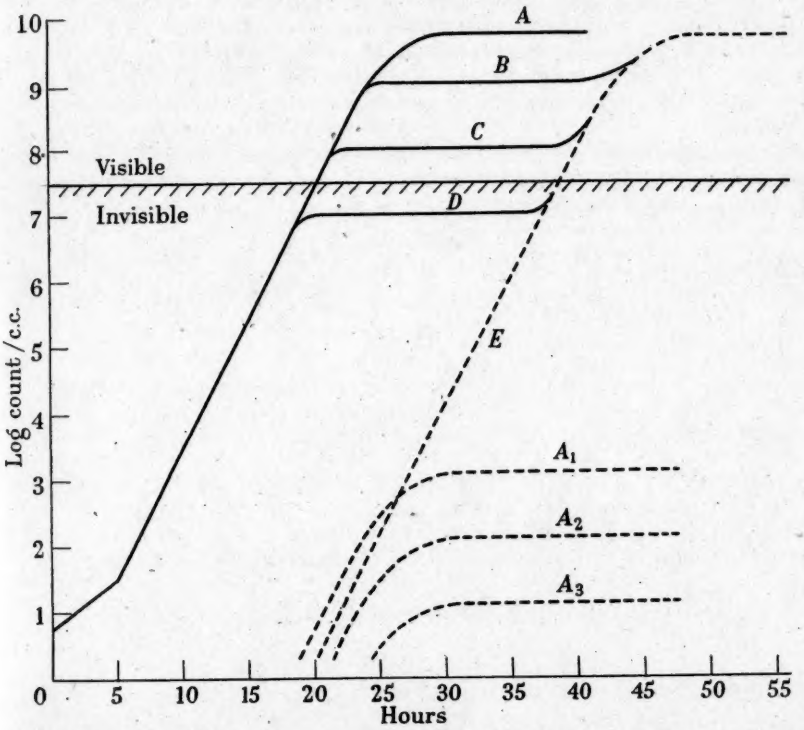

FIG. 5.—Effect of tryptophan on growth of mutants.

———— = total count ; - - - - - - = mutants.

A = peak of viable count of a culture unlimited by inadequacy of tryptophan.

A1, A2, A3 = growth curves of any mutants which happen to appear in cultures like A when they have reached 10^7, 10^8 or 10^9 bacteria/c.c. respectively. A1, A2 and A3 are equivalent to that part of curve A which is drawn above the point of occurrence of the mutant. This only is available for growth of the mutant together with the parent. Both grow for the same number of generations, 9 in the case of A and A1.

B is a culture with a peak of growth limited to 10^9 bacteria/c.c. by deficiency of tryptophan. C is another culture limited to 10^8/c.c. D is limited to 10^7/c.c. Each one of these cultures is assumed to have mutated at 10^7/c.c. E represents the growth of these mutants which overtakes the limited cultures and carries the total count up to the limit. Culture D had not grown visibly, and in this case the whole of the visible growth is due to mutants.

to attain 10^7 bacteria/c.c., and then if mutation occurred, another four days might elapse before the mutant had grown to a visible extent. The bacteria of Fildes, Gladstone and Knight suffered from many hindrances other than a shortage of tryptophan, and so gave the impression of growing after a long period of stagnation, but the bacteria of the present work enjoyed reasonably satisfactory growth conditions, which lead to the detection of mutation in a much shorter time.

CONCLUSIONS.

1. Units of *Bact. typhosum*, capable of growing without tryptophan, are constantly present in normal cultures which require tryptophan.
2. Their appearance is unrelated to the concentration of tryptophan in the culture.
3. This finding is in accordance with expectation if the units are mutants of genetic origin.
4. It follows that " training " bacteria to dispense with certain nutritive substances normally essential may be looked upon as a cumbersome method for selecting genetic mutants.

REFERENCES.

FILDES, P.—(1945) *Brit. J. exp. Path.*, **26**, 416.
Idem, GLADSTONE, G. P., AND KNIGHT, B. C. J. G.—(1933) *Ibid.*, **14**, 189.
LURIA, S. E.—(1947) *Bact. Rev.*, **11**, 1.
MONOD, J.—(1946) *Ann. Inst. Pasteur*, **72**, 879.
WOLLMAN, E., jun.—(1947) *Ibid.*, **73**, 1082.
ZAMENHOF, S.—(1946) *J. Bact.*, **51**, 351.

EXPERIMENTAL NEPHRITIS: AN ATTEMPT TO DEMONSTRATE A NEPHROTOXIC SUBSTANCE IN THE SERUM OF ACUTE NEPHRITICS.

R. READER.*

From the Department of Medicine, University of Sydney.

Received for publication May 6, 1948.

IN 1941 Schober reported an experiment, the conclusions of which were of fundamental importance in the study of the aetiology of acute nephritis. The author reviewed the evidence in favour of an allergic mechanism of aetiology, and considered that it was by no means conclusive. He claimed that the main factor in this evidence was the lag period between the preceding infection and the onset of nephritis, but pointed out that this could be just as readily explained, if the renal lesion were due to bacterial endotoxin liberated after the infection had subsided, when great numbers of bacteria were undergoing lysis.

In support of this concept he claimed to have demonstrated a toxic substance in the serum of patients suffering from active nephritis which could produce acute nephritis in rats. Further, he demonstrated that the substance was not a

* Marion Clare Reddall Research Scholar.

protein, as protein-free ultra-filtrates of the serum produced the nephritis just as readily. He concluded, therefore, that antibody mechanisms were not involved.

Serum from cases of active acute nephritis, healed acute nephritis, active chronic nephritis, quiescent chronic nephritis, essential hypertension and toxaemia of pregnancy was investigated. Only serum from active acute nephritis and active chronic nephritis produced renal lesions in the experimental animals. If this observation is confirmed it must produce considerable modification of most modern concepts of the aetiology and pathogenesis of acute nephritis.

Several criticisms of Schober's work seem warranted. In the first place the criterion of diagnosis was histological, and no observations were made on the living rats. It is doubtful if it is justifiable to diagnose acute nephritis in a rat on histological data alone, and more conclusive evidence would be required that the treated rats developed a true acute nephritis before conclusions concerning acute nephritis in man could be drawn.

Although the description of the lesions conforms to the commonly accepted changes in acute nephritis, the photomicrographs in some instances do not support the descriptions. Thus an increase in nuclei of the glomerular tuft is described in Fig. 1, 2, 4 and 5 ; yet a count reveals numbers varying from 47 to 101. No indication is given of the normal, but in my material these findings would be within the normal range. Further, unless observations are made on the living rats prior to treatment, it is difficult to exclude spontaneous renal disease in a small group of animals. Finally, many of Schober's rats were infested by a protozoan parasite, *Klossiela muris*, in the tubular epithelium of the kidneys. Although he claims that this organism does not cause kidney damage, he admits that its occurrence detracts from the value of the experiment.

The following is a report of an unsuccessful attempt to reproduce Schober's results.

MATERIAL.

Blood was obtained from 8 cases admitted to the Royal Prince Alfred Hospital or The Royal Alexandra Hospital for Children, Sydney, clinically diagnosed as active acute nephritis.

Fully grown Wistar strain male rats were used throughout.

METHOD.

As soon as possible after admission 10 to 30 ml. of venous blood were obtained by sterile syringe, and allowed to clot at room temperature in a sterile centrifuge tube. In the first experiment the serum so obtained was divided, some being injected whole into a rat, the remainder being passed through a collodion filter prepared as described by Schober (1941). The ultrafiltrate, which gave negative reactions to Biuret and Tsuchiya's reagents, was injected into a second rat.

As the results of this experiment were negative, it was decided to omit the ultrafiltration in subsequent experiments, in order that each rat should be given as large a dose as possible. Schober emphasizes the importance of giving a large amount of serum in proportion to body weight of the rat.

The last seven sera were therefore given whole to the test rats, in amounts varying from 3·5 ml. to 12 ml. The injections were all subcutaneous.

The rats were kept in round metabolism cages and fed on a ration of compressed pellets specially prepared for rats. A series of preliminary observations

of weight, urine and blood pressure was made on each rat for periods varying from two weeks to several months prior to experiment.

This preliminary period of observation was necessary, as many of the untreated animals were found to pass protein in the urine to the extent of 0·01 to 0·05 g. per cent and had to be discarded. Proteinuria was noticeable in almost all rats when first separated and placed in metabolism cages, and may have been due to psychic or temperature factors. After about 7 days the urine usually cleared and remained clear, but in some the proteinuria persisted during many months of observation. The latter animals were not used for the experiments.

The source of this protein was not traced. Many rats were found to be infested by a bladder worm, *Trichosomoides crassicauda* (Hall, 1916 ; Yokogawa, 1921), and it was thought that irritation in the bladder may have accounted for the proteinuria. However, no direct correlation between proteinuria and bladder infestation could be demonstrated in a number of autopsy examinations.

The urine was collected overnight, a 16-hour period, during which food was removed from the cage. It was measured and examined quantitatively by Aufrecht's method for protein, and microscopically for formed elements.

Blood pressures were estimated by the plethysmograph method of Byrom and Wilson (1938), the animal being under ether anaesthesia.

After treatment, the weight, blood pressure and urine observations were made as frequently as possible, usually at about 2-day intervals, and the animal was sacrificed by an overdose of ether after 6 to 14 days.

The kidneys were examined macroscopically, then split and fixed in Zenker's formaldehyde solution or 10 per cent formol saline. Sections were stained by haematoxylin and eosin and by Masson's haematoxylin-ponceau acid fuchsin—light green method.

Preliminary observations on untreated rats.

1. *Urines.*—The amount collected in a 16-hour period varied from 3 to 15 ml. As indicated above, many of the rats passed significant amounts of protein in their urine. Although these animals showed no organized deposit in the urine, and no hypertension, they were discarded. Most samples contained heavy deposits of ammonium magnesium phosphate crystals. Very occasionally one or two erythrocytes were noted in a specimen, but no animal in the series showed significant numbers of red cells or casts.

2. *Weights.*—The weights at the time of injection varied from 140 g. to 270 g.

3. *Blood pressures.*—The blood pressures varied from 90 to 130 mm. of mercury, with an occasional isolated reading as low as 70 mm. or as high as 150 mm. of mercury.

4. *Histology.*—Histological observations on untreated and apparently normal rats showed the glomerular tufts were filled with blood, their capillary lumina patent and containing from 40 to 90 nuclei. Bowman's space was free from débris and was 15 to 20 μ in width. There was no evidence of adhesions, and the capsule consisted of a thin layer of flattened cells, lying on a thin basement membrane continuous with that of the tubules, and staining a bright green with Masson's technique.

Many of the tubules appeared broken up or distorted, but in the intact ones

the lumina were free of debris and there was no evidence of degenerative changes in the cells. Each convoluted tubule was surrounded by a basement membrane of green-staining collagenous material, apart from which the cortex was free from connective-tissue elements.

RESULTS.

The case-histories and investigations on the corresponding rats are given in brief. It may be stated, however, that in no instance did the rats show any evidence, either clinical or histological, of renal disease.

Case 1.—*L. W—. Male, aged* 11 *years.*

He had suffered two severe attacks of rheumatic fever previously. He also suffered frequently from colds prior to the onset of present illness. The onset was marked by aching in the knees and back and, two days later, swelling of the face, feet and hands. The swelling was subsiding when, 8 days later, the urine became dark. He was admitted on the 14th day of illness with puffiness of the face and swelling of the feet. The blood pressure was 160/105. There was moderate cardiac enlargement, and a systolic murmur was audible in all areas. The urine showed macroscopic haematuria, proteinuria, and hyaline and granular casts. The blood urea was 29 mg. per cent. The oedema subsided rapidly, but mild hypertension and haematuria persisted till the patient was discharged to bed at home on the 53rd day of the illness.

On the 15th day of illness 15 ml. of blood were withdrawn at 12 midday. 7·5 ml. of serum were collected, and 2 ml. of whole serum were injected subcutaneously into rat 8. From the other 5 ml. 2·5 ml. of water-clear, protein-free ultrafiltrate were obtained and injected into rat 10. The injections were made 4½ hours after collection of the blood.

Rat 8.—Had been observed for 3 weeks prior to the experiment. The urine contained no abnormal constituents. Weight 139 g. Blood pressure 114 mm. of mercury. Given 2 ml. of whole serum subcutaneously. There were no immediate effects. For the following 6 days the rat passed normal amounts of urine which was protein free, and free from organized deposit. The blood pressure varied from 110 to 125 mm. of mercury, and the weight fell to 135 g.

Killed on the sixth day.

Sections of the kidney showed no deviation from the normal. In particular, there was no swelling of the tuft and no increase in nuclei. There was no increase in collagenous material in the tuft or interstitial regions, and no exudate in Bowman's space nor in the lumina of the tubules.

Rat 10.—Had been observed for 3 weeks prior to experiment. The urine contained no abnormal constituents. Weight 139 g. Blood pressure 115 mm. of mercury. 2·5 ml. of serum ultrafiltrate given. The rat remained well, passed urine in normal amounts and free from protein and organized deposit. Weight increased to 159 g. but fell to 139 g., by the 6th day. The blood pressure remained unaltered.

Killed on the 6th day.

Kidney sections showed no evidence of pathological change. Glomerular nuclei counts varied from 50 to 80.

Case 2.—*M. G—. Male, aged* 3½ *years.*

Mild sore throat 4 weeks prior to illness. Onset with vomiting, malaise, backache, frequency of micturition and diminished output of urine, dark urine, and slight puffiness of the face. Blood pressure 125/95. Blood urea 32 mg. per cent. Urine showed macroscopic haematuria and proteinuria. The illness was prolonged, and the patient was discharged on the 110th day symptom-free and with normal urine.

3·5 ml. of whole serum collected on the 4th day of illness were injected subcutaneously into Rat 1, within 4 hours of collection.

Rat 1.—Had been observed for 5 months prior to experiment. Urine was free from protein and organized deposit. Weight 179 g. Blood pressure had varied from 95 to 135 mm. of mercury. 3·5 ml. of whole serum given. No immediate ill effects, and the rat remained apparently well for the next 8 days. Urine was passed in normal amounts and was free from protein and organized deposit. Weight fell to 160 g., and blood pressure varied from 90 to 125 mm. of mercury.

Killed on the 8th day.

Sections of the kidney showed no abnormality.

Case 3.—*P. C—. Male, aged* 5 *years.*

Impetigo of arms and legs for 14 days. Illness commenced with vomiting. Two days later the face became swollen. Admitted on the 4th day with puffiness of the face, microscopic haematuria, slight proteinuria and hyaline and granular casts. Blood pressure 170/120. Blood urea 25 mg. per cent. Recovery was rapid, and he was discharged on the 49th day of illness.

6 ml. of whole serum collected on the 5th day of illness were injected subcutaneously into rat 5, 3½ hours after collection.

Rat 5.—Had been observed for 5 months prior to the experiment. Urine was free from protein and organized deposit. Blood pressure had varied from 90 to 125 mm. of mercury, with one isolated reading of 150 mm. Weight 182 g. 6 ml. of whole serum given. The animal remained apparently well for the next 14 days. Blood pressure 105 mm. of mercury, weight 180 g. Urine was passed in normal amounts and was free of protein and organized deposit.

Killed on 14th day.

Kidney sections showed no evidence of pathological changes.

Case 4.—*P. C—. Male, aged* 5 *years.*

Sore throat for 2 weeks prior to illness. Onset with dark urine and frequency of micturition, and on the following day puffiness of the face. Admitted on the second day of illness with slight oedema of the face, a trace of protein in the urine, microscopic haematuria, and occasional hyaline and granular casts. Blood pressure 115/70. Symptoms subsided rapidly, and the urine was normal by the 41st day.

6 ml. of serum collected on the 6th day of illness were injected subcutaneously into rat 11, 6 hours after collection.

Rat 11.—Had been observed for 2½ months prior to the experiment. Urine was free from protein and organized deposit. Blood pressure had varied from 105 to 135 mm. of mercury. Weight 172 g. 6 ml. of whole serum given. The

rat collapsed during the injection and remained unconscious, with rapid shallow breathing, for several minutes. It recovered rapidly and remained well for the remainder of the observation period. No subsequent observations were made on the weight or blood pressure or urinary protein. On the day after injection occasional erythrocytes were noted in the urine. Subsequently the urine was free from organized deposit.

Killed on the 6th day.

Kidney sections showed no evidence of renal lesions.

Case 5.—*M. L—. Female, aged* 11 *years.*

Generalized impetigenous lesion and discharging ear for some weeks. Onset with vomiting and rigors. Next day, swelling of the face, oliguria and dark urine. Admitted on the 6th day showing no oedema but microscopic haematuria, heavy proteinuria, and hyaline and granular casts. Blood pressure 115/85, and moderate cardiac enlargement. No change till the 19th day, when an exacerbation occurred lasting till the 51st day, the blood pressure rising to 160/105 and the urine becoming frankly blood stained. On the 61st day a second exacerbation occurred, lasting till the 72nd day. From then gradual improvement till time of discharge, when the urine still showed a trace of protein and microscopic haematuria.

9·5 ml. of serum, collected on the 9th day of illness, were injected subcutaneously into rat 19, 3 hours after collection.

Rat 19.—Had been observed for 2½ months prior to the experiment. Urine was free from protein and organized deposit. Blood pressure had varied from 90 to 150 mm. of mercury. Weight 190 g. 9 ml. of whole serum given. No immediate effect, and the rat remained apparently well. Urine was passed in normal amounts, and was free from organized deposit and from protein. Blood pressure varied up to 130 mm. of mercury, and weight fell to 175 g.

Killed on the 13th day.

Kidney sections showed some areas in which the glomerular tufts appeared swollen, completely filling Bowman's space. In these areas the tufts contained 80 to 100 nuclei. There was no evidence of capsular adhesions, no proliferation of the capsular epithelium, and no exudate in the capillary lumina, which were patent and filled with blood. Tubule cells were also swollen and the lumina obliterated. The greater part of the cortex appeared normal.

Case 6.—*R. B—. Male, aged* 9 *years.*

Impetigenous lesions on hands and feet for 1 month prior to illness. Onset with swelling of the face, abdomen, hands and legs. Urine diminished and smoky. Admitted on approximately the 14th day of illness, with massive generalized oedema, blood pressure 140/90, heavy proteinuria, microscopic haematuria, and hyaline, granular, and epithelial casts. Proteinuria, sometimes as high as 2 g. per cent, and oedema persisted. Blood urea varied from 40 to 90 mg. per cent. Repeated abdominal paracenteses were necessary, and the patient died on the 96th day of illness.

4·5 ml. of serum collected on the 14th day of illness were injected subcutaneously into rat 38, 3 hours after collection.

Rat 38.—Had been observed for 2 weeks prior to the experiment. Urine

was free from protein and organized deposit. Blood pressure 70 mm. of mercury. Weight 205 g. 4·5 ml. of whole serum given. There was no untoward reactions and the rat remained apparently well. Urine was passed in normal amounts, and remained free of protein and organized deposit. Blood pressure varied from 65 to 115 mm. of mercury. Weight rose to 220 g. and remained constant.

Killed on the 12th day.

Kidney sections showed generalized swelling of the glomerular tuft, so that there was very little remaining of the capsular space. In some glomeruli the space was obliterated in part, suggesting capsular adhesions, but there was no other evidence of inflammatory change. The capillary lumina were patent, there was no increase in nuclei of the tufts, no capsular proliferation, and no evidence of tubular degeneration nor cast-formation.

In view of the lack of evidence of any pathological change, apart from swelling of the tufts, in rats 19 and 38, the appearance was considered to be due to a fault in fixation of the kidney tissue.

Case 7.—*A. B—. Male, aged* 18 *years.*

"Cold" for 1 month. Dark urine for 3 weeks and swollen face and ankles for 2 weeks. Admitted on 21st day of illness with generalized oedema and blood pressure 155/105. Urine showed microscopic haematuria, massive proteinuria, and hyaline and granular casts. Oedema subsided slowly, but hypertension, haematuria and heavy proteinuria persisted till discharge.

12 ml. of serum collected on the 21st day of illness were injected subcutaneously into rat 40, 1¾ hours after collection.

Rat 40.—Had been observed for 2 weeks prior to experiment. Urine had constantly shown less than 0·01 per cent. protein, and was free from organized deposit. Weight 272 g. Blood pressure 95 mm. of mercury. 12 ml. of whole serum given. The rat collapsed during injection and remained unconscious for one minute, but recovered and remained apparently well. Urine was passed in normal amounts and was free from protein and organized deposit. Weight fell to 248 g., and blood pressure varied from 85 to 120 mm. of mercury.

Killed on the 12th day.

Kidney sections showed no pathological changes.

Case 8.—*T. M—. Male, aged* 4½ *years.*

An attack of tonsillitis 3 weeks prior to onset. Face became swollen, with vomiting and headache, and two days later dark urine was noted. Admitted on 3rd day of illness, drowsy, with oedema of face and lumbar tenderness. Blood pressure 110/?. A soft mitral systolic murmur was present. Blood urea 80 mg. per cent. Urine frankly bloodstained, with moderate proteinuria. Symptoms subsided, but microscopic haematuria persisted during the period of observation.

5 ml. of serum collected on the 5th day of illness were injected subcutaneously into rat 39, 3½ hours after collection.

Rat 39.—Had been observed for 3 weeks prior to experiment. Urine had consistently contained less than 0·01 per cent of protein and was free from organized deposit. Blood pressure 100 mm. of mercury. Weight 242 g. 5 ml. of whole serum given. No immediate reaction, and the animal remained apparently well. Urine was passed in normal amounts and was free from protein

and organized deposit. Weight remained constant. Blood pressure 120 mm. of mercury.

Killed on the 7th day.

Kidney sections showed no abnormality.

SUMMARY.

An unsuccessful attempt to demonstrate a nephrotoxic substance in the serum of patients suffering from acute nephritis is reported.

The demonstration of such a substance had been claimed by Schober in 1941.

Sera from 8 patients clinically diagnosed as acute nephritis were injected subcutaneously into rats. Subsequently the animals showed no evidence of renal disease, either during life or on histological examination of the kidneys.

The co-operation of the honorary and resident Medical Staff of the Royal Prince Alfred Hospital and Royal Alexandra Hospital for Children, Sydney, in making their cases available, is gratefully acknowledged.

I should also like to express my thanks to Dr. F. B. Byrom for considerable assistance and advice on the care and investigation of the rats.

REFERENCES.

Byrom, F. B., and Wilson, C.—(1938) *J. Physiol.*, **93**, 301.
Hall, M. C.—(1916) *Proc. U.S. nat. Mus.*, **50**, 13.
Yokogawa, S.—(1921) *J. Parasit.*, **7**, 80.
Schober, B.—(1941) *Edinb. med. J.*, **48**, 322.

SERUM COMPLEMENT IN ACUTE NEPHRITIS.

R. READER.*

From the Department of Medicine, University of Sydney.

Received for publication May 6, 1948.

IT is desired, in this communication, to draw attention to previous work on changes in serum complement titre in acute nephritis and to report a series of observations in confirmation.

The subject of serum complement has become more and more complex as a result of modern methods of protein chemistry, but for present purposes it may be defined as a normal constituent of serum, which is necessary for the completion of most antigen-antibody reactions, and which is used up, or fixed, as a result of such a reaction. The level in normal individuals is remarkably constant, and is not affected by age or sex (Ecker, Seifter and Duzois, 1945; Rutstein and Walker, 1942; Pohl and Rutstein, 1944).

Variations from the normal occur in a number of conditions. It was considered originally that such changes may be an indication of variation in immunity, but observations on patients suffering from infectious diseases have been inconclusive. During the acute stages the majority of cases show an increase, which

* Marion Clare Reddall Research Scholar.

tends to fall to normal levels with recovery. In some fulminating cases the level falls very low.

Some diseases of suspected anaphylactic nature have been investigated. Coburn (1936) found a fall in the titre of complement in cases of serum sickness, though he gives no details, and Rutstein and Walker (1942) noted a fall in six of eleven patients developing serum sickness after antiserum treatment of pneumonia. Similarly, in acute rheumatic fever, Coburn (1936), Veil and Buchholz (1932) and Rachmilewitz and Silberstein (1937) have noted a fall in titre, which they consider a means of differentiating the condition from acute rheumatoid arthritis.

Gunn (1914) noted that in four cases of acute nephritis complicating scarlet fever, complement was almost absent from the serum. Veil and Buchholz (1932) comment on the marked fall in acute glomerulo-nephritis, and therefore infer that the disease is a variant of rheumatic fever.

Kellett (1936) reports a marked fall in serum complement titre in three cases of acute nephritis. He used a technique wherein the normal figure was 100 per cent. In all three cases the initial titre was less than 20 per cent, and each rose to normal as recovery occurred. In a control series of acute streptococcal infections both at the height of infection and during convalescence the complement levels were normal, varying from 80 per cent to 90 per cent. Kellett postulates that the complement in nephritis patients has been used up during an antigen-antibody reaction, a manifestation of reversed anaphylaxis. Kellett and Thomson (1939) using the same method studied 26 cases of nephritis. In 16 acute cases the initial complement titre was low in every case, varying from 0 to 42 per cent. In two other cases, diagnosed clinically as acute nephritis, the complement levels were normal, and in each case autopsy revealed no acute nephritis. In the majority of cases the titre rose to normal in three to four weeks. In five it remained low ; three of these proved fatal, while two made apparently complete recoveries in spite of the low complement. In four subacute cases the figures tended to be low, 72, 64, 70, 52 per cent, while in six chronic cases the readings were all over 80 per cent. The authors suggest that the test is of diagnostic value, and quote the two cases clinically diagnosed as acute nephritis in whom the normal complement level cast a doubt which was confirmed at autopsy. Secondly, they suggest that the test may serve to differentiate those cases which present as acute nephritis but are, in fact, a progressive stage of a previously undiagnosed attack. Thirdly, they suggest that it may be a means of determining whether chronic nephritis is the result of a single progressive attack of acute nephritis, or of multiple recurrences. They consider that the observations probably have little prognostic significance, as the outcome is just as clearly apparent by clinical criteria.

Thomson, Arnott and Mathew (1939) report similar findings in 16 of 18 cases of acute nephritis. Observations on 126 normal persons, 157 normal pregnant women, 33 cases of pre-eclamptic toxaemia and 8 cases of eclampsia with convulsions, showed the complement titres to be normal. No details of the results are given.

MATERIAL.

The following observations have been made on 16 cases clinically diagnosed as acute nephritis, admitted to the Royal Prince Alfred Hospital or the Royal Alexandra Hospital for Children during the years 1946 and 1947. The first

sample of serum was taken as early as possible after admission, and where possible serial estimations were made.

A control series of 47 individuals, normal or suffering from illnesses of non-anaphylactic or infective nature, was investigated at the same time.

METHOD.

Five ml. of blood were withdrawn by sterile paraffined syringe, and allowed to clot. Estimations were made on the serum on the day of collection, except in some of the control series, which were stood in the refrigerator overnight and estimated within 24 hours of collection. Preliminary investigation had revealed that there was no loss of complement activity under these conditions for 24 hours. This is in agreement with the results of Pohl and Rutstein (1944).

Doubling dilutions of serum in 1 c.c. amounts were run out in 3/8 in. tubes with a range of 1/1 to 1/256. To each tube 0·01 ml. of packed, sensitized sheep's erythrocytes were added to make approximately a 1 per cent suspension.

The sensitized cells were prepared as follows :

> Fresh washed sheep's erythrocytes were obtained from the Fairfax Institute of Pathology, Royal Prince Alfred Hospital, and 1 ml. of packed cells was added to 20 ml. of 0·9 per cent saline to which 0·2 ml. of glycerinated haemolysin (Commonwealth Serum Laboratory—titre 1/5000) had been added. After standing for 30 minutes at room temperature, the tube was centrifuged for 10 minutes at 2000 r.p.m., and the supernatant saline poured off. These cells were stored in a refrigerator and used for a week from the time of collection.

Four control tubes were set up as follows :

(1) 1 ml. 0·9 per cent saline plus 0·01 ml. sensitized cells.
(2) 1 ml. 0·9 per cent saline plus 0·01 ml. normal cells.
(3) 1 ml. 1/20 dilution of serum plus 0·01 ml.normal cells.
(4) 1 ml. 1/20 dilution of serum (heated at 56°C. for 10 minutes) plus 0·01 ml. normal cells.

The tubes were placed in a water bath at 37°C. for 30 minutes, then stored overnight in a refrigerator, and the reading was made next morning. Complete haemolysis was taken as the end point.

In the nephritis cases a parallel series from a normal control, and in many cases from the same individual, were set up simultaneously.

RESULTS.

The results were expressed as the reciprocal of the serum dilution producing complete haemolysis. The majority of the control series contained enough complement to produce complete haemolysis in the 1/32 dilution. The serum was thus considered to contain 32 minimal haemolytic doses (M.H.D.) of complement per ml.

In the control series the titre ranged from 16 to 256 M.H.D. per ml., with the great majority 32 or 64. The distribution is indicated in the histogram (Fig. 1).

The case histories and complement estimations of 16 acute nephritics are

summarized in Table I. The distribution of initial complement estimation is compared with the control series in the histogram (Fig. 1). The graph (Fig. 2) shows serum complement levels correlated with the day of illness in each case.

In 11 cases the initial readings were well below the normal range, and in

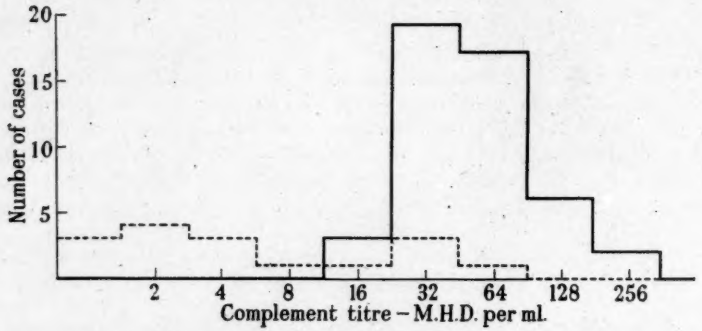

FIG. 1.—Distribution of normal series indicated by continuous line. Initial estimation of nephritic series indicated by discontinuous line.

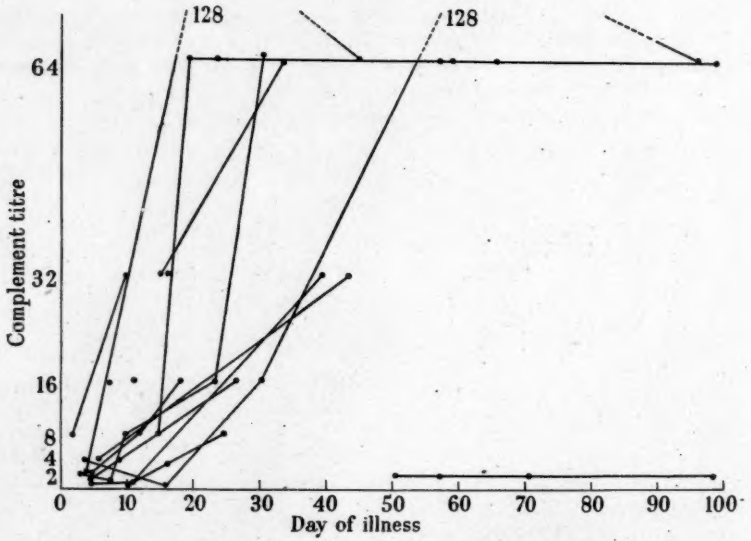

FIG. 2.—Serial complement titres in eleven cases of acute nephritis.

9 markedly so. In 5 the initial reading fell within the normal range. It is probable that in these cases the titre had already risen. It is apparent from Fig. 2 that the titre may rise from a very low figure to normal within 10 to 20 days of onset. For example, in Case 8 the titre rose from 2 on the fourth day to 128

TABLE I.

No.	Age.	Sex.	Clinical history.	Day of illness.	Complement MHD/ml.
1. P.S—	8½	F.	Intermittent sore throats for 6 to 8 weeks. Pallor; puffy face, aching hips and shoulders and smoky urine five days prior to admission. On admission no oedema. B.P. 140/95. Proteinuria, haematuria and hyaline casts. Blood urea 42 mg. per cent. Recovery rapid, and discharged on 31st day of illness	10 17 24	1 4 8
2. R. S—	11	M.	Headache, nausea and vomiting 10 days before. Well for 1 week, then headache, vomiting, swollen face and hands and dark urine. Admitted on 4th day with oedema. B.P. 165/105, haematuria, proteinuria and granular casts. Recovery rapid, and discharged on the 25th day of illness	5 12 18	2 8 16
3. J. McD—	9	M.	Measles three weeks prior to admission and impetigo present for one month. Swelling of face and wrists. No vomiting, no headache and no urinary symptoms. Admitted on 3rd day of illness with oedema of face. B.P. 150/110, haematuria, proteinuria and epithelial and granular casts. Blood urea 47 mg. per cent. Discharged on 42nd day of illness	5 11 39	1 1 32
4. F. S—	47	M.	Cough, dry throat, headache and backache for two days. Well for three days, then swelling of face, feet, legs and genitalia. Admitted on day of onset with generalized oedema. B.P. 170/100. Proteinuria and haematuria. Blood urea 102. Oedema and B.P. subsided rapidly, but slight haematuria and cylindruria persisted at time of discharge on 29th day of illness	2 10	8 32
5. L. W—	11	M.	Had suffered two severe attacks of rheumatic fever previously. Frequent colds prior to present illness. Aching in knees and back, two days later swollen face, feet and hands. Swellings were subsiding when, eight days later, the urine became dark. He was admitted on 14th day of illness, with puffy face and feet, B.P. 160/105, moderate cardiac enlargement and a systolic murmur in all areas. Urine showed macroscopic haematuria, proteinuria and hyaline and granular casts. Blood urea 29 mg. per cent. Oedema subsided rapidly, but mild hypertension and haematuria persisted till discharged to bed at home on 53rd day of illness	15 33	32 64

TABLE I. (*cont.*)

No.	Age.	Sex.	Clinical history.	Day of illness.	Complement MHD/ml.
6. M. G—	3½	M.	Mild sore throat four weeks prior to onset. Vomiting, malaise, backache, frequency and diminished output of urine, dark urine and slight puffiness of face. Admitted on 2nd day of illness with pallor and puffiness of face, B.P. 125/95 and blood urea 32 mg. per cent. Urine showed macroscopic haematuria and proteinuria. The illness was prolonged, and patient discharged on 110th day of illness. Symptom-free and with clear urine	4 16 30 57 95	4 1 16 128 64
7. P. C—	5	M.	Impetigo of arms and legs for 14 days prior to onset. Illness commenced with vomiting, and two days later face became swollen. Admitted on 4th day with puffiness of face, microscopic haematuria, slight proteinuria and hyaline and granular casts, B.P. 170/120. Blood urea 25 mg. per cent. Recovery was rapid, and he was discharged on 49th day after tonsillectomy on 32nd day	5 26	1 16
8. P. C—	5	M.	Sore throats for two weeks prior to illness. Noted dark urine and frequency, and puffy face the following day. Admitted on 2nd day of illness with slight oedema of face, trace of protein in urine, microscopic haematuria and occasional hyaline and granular casts. B.P. 115/70. Symptoms subsided rapidly, and urine was clear by the 41st day	4 18 45	2 128 64
9. M. L—	11	F.	Generalized impetigenous lesions and discharging ear for some weeks. Onset with vomiting and rigors. Next day swelling of face, oliguria and dark urine. Admitted on 6th day showing no oedema, microscopic haematuria, heavy proteinuria and hyaline and granular casts. B.P. 115/85 and some cardiac enlargement. No change occurred till 19th day, when an exacerbation occurred, lasting till the 51st day. B.P. rising to 160/105 and urine becoming frankly blood-stained. On 61st day a second exacerbation occurred, lasting till 72nd day. From then gradual improvement till time of dischage, when urine still showed a trace of protein and microscopic haematuria. In this patient serum complement initially low, rose to normal, and remained at a normal level throughout two clear-cut exacerbations	9 15 19 23 37 58 60 65 108	4 8 64 64 64 64 64 64 64
10. R. B—	9	M.	Impetigenous lesions on hands and feet for one month prior to illness. Onset with swelling of face, abdomen, hands and legs. Urine diminished and smoky during this time. Admitted on approximately 14th day of illness with massive generalized oedema. B.P. 140/90. Massive proteinuria, microscopic haematuria and hyaline granular and epithelial casts. Proteinuria at times as high as 2 per cent and oedema persisted. Blood urea varied from 40 to 90 mg. per cent. Repeated abdominal paracenteses were necessary and patient died on 96th day of illness	14	32

TABLE I (*cont.*).

No.	Age.	Sex.	Clinical history.	Day of illness.	Complement MHD/ml.
11. A. B—	18	M.	Had a common cold for one month, dark urine for three weeks and swollen face and ankles for two weeks. Admitted on 21st day of illness with pitting oedema of ankles and legs and oedema of face. B.P. 155/105. Urine showed microscopic haematuria, massive proteinuria and hyaline and granular casts. Oedema subsided slowly, but haematuria and heavy proteinuria persisted until discharge	21	64
12. M. H—	1½	F.	Attack of coryza had settled down for one week when swelling of legs and abdomen was noted. Admitted on 7th day of illness with generalized oedema, heavy proteinuria and microscopic haematuria. Condition subsided, and patient discharged on 41st day	7	16
13. C. J—	5½	M.	A sore throat had subsided for three weeks when haematuria was noted, and this persisted without oedema or constitutional symptoms till admission on approximately the 50th day of illness. At this time there was slight puffiness of face B.P. 155/100. moderate proteinuria and microscopic haematuria. Condition remained unchanged during the period of observation	50 57 70 106	2 2 2 2
14. T. M—	4½	M.	An attack of tonsillitis three weeks prior to onset. Face became swollen, with vomiting and headache, and two days later dark urine was noted. Admitted on 3rd day of illness, drowsy, with oedema of face and renal tenderness. B.P. 110/? and a soft mitral systolic murmur. Blood urea 80 mg. per cent; urine frankly bloodstained, with moderate proteinuria. Symptoms subsided but haematuria persisted during period of observation	4 8 10 23 30	2 2 8 16 64
15. E. G—	4	M.	Listless and drowsy and one day later noted red urine. Admitted on second day of illness with B.P. 115/60, no oedema, blood urea 78 mg. per cent, heavy proteinuria, macroscopic haematuria and granular casts. Illness was mild, and patient was discharged on 28th day	8	32
16. J. W—	4	M.	Tonsillectomy ten weeks and coryza two weeks prior to onset. Illness commenced with red urine and three days later nausea and vomiting. Admitted on 4th day of illness. Tonsillar fossae inflamed and cervical glands enlarged. No oedema. B.P. 110/60. Macroscopic haematuria, proteinuria, no casts. Blood urea 50 mg. per cent. Renal symptoms subsided rapidly in spite of intercurrent measles and pneumonia	6 43	4 32

on the eighteenth, and in Case 4 from 8 on the second day to 32 on the tenth. It is thus apparent that interpretation of an isolated estimation will depend on the interval since the onset of symptoms.

DISCUSSION.

In the present series it does not seem possible to draw conclusions as to a relationship between the severity of nephritis and the degree of fall in complement titre. In Case 8 the nephritis was very mild, yet the initial complement estimation, on the fifth day, was 4. Similarly, in Case 16 the initial titre was 4 on the sixth day. On the other hand, Case 9 was prolonged with two clear-cut and severe exacerbations, yet the initial reading of 4 had risen to 64 on the 19th day, and remained at that level throughout both exacerbations.

It is difficult to understand why the complement level was unaffected by the latter two episodes. It seems logical to assume that they were the result of the same mechanism operating in the initial attack. If Kellett's hypothesis, which is widely held, is correct, then the patient suffered two further anaphylactic reactions without measurable fixation of complement.

It is possible that the answer lies in the power of the body to restore complement, a factor probably independent of the nephritis. Having been stimulated by the initial fall, a vigorous response might be capable of maintaining the normal level in spite of repeated anaphylactic reactions. The lack of such a response would explain the persistently low titre in Case 13. Although not investigated until approximately the 50th day of illness, the complement level was 2, and remained at this level until last examined on the 106th day. Yet, during this time there had been no changes suggesting repeated anaphylactic episodes.

Previous writers have indicated that the level of serum complement is not dependent on the degree of proteinuria, or the level of plasma protein. Cases 9, 10 and 11 all showed massive proteinuria and hypoproteinaemia at a time when serum complement titres were normal. On the other hand, Cases 1, 3, 7 and 8, with considerable fall in complement, showed only slight proteinuria at all times.

The phenomenon has theoretical and practical application. In the first place it seems to indicate an anaphylactic mechanism in pathogenesis, and this is an important link in the chain of evidence for such a theory of aetiology. Veil and Buchholz (1932) and Rachmilewitz and Silberstein (1937) refer to experimental work in which anaphylactic reactions in animals were accompanied by a fall in complement titre, and similar findings in patients suffering from serum sickness have been referred to above.

The technique is simple enough to be of diagnostic aid in doubtful cases. In view of the rapid rise to normal in some cases, a normal titre would be of little value in excluding the diagnosis unless taken within a few days of the onset of symptoms. On the other hand, a low titre at any stage would be of considerable assistance in confirming the diagnosis.

The lack of exact criteria for the diagnosis of acute nephritis has rendered the investigation of aetiology exceedingly difficult, particularly in the experimental field, where it is difficult to be sure that a condition produced in a group of animals is equivalent to the human disease. It is submitted here that a fall in complement titre is characteristic of the disease, and should be used as a further criterion before diagnosing acute nephritis in treated animals.

The test may also be of use to decide whether differentiation of acute focal from acute diffuse nephritis is justified. The former condition is considered to be due to lodgement of bacteria from an infected focus, in the capillary loops of the glomerular tufts, thereby producing haematuria and proteinuria without hypertension, nitrogen retention or oedema. An anaphylactic mechanism is not involved, and the condition is considered fundamentally different from acute diffuse nephritis. The importance of making a differentiation is considerable, as the focal form is considered to have an entirely benign prognosis. However, there is a growing tendency to doubt whether the focal condition differs in anything but degree from the diffuse, and Payne and Illingworth (1940) bring considerable evidence in support of such a concept. This view is becoming prevalent probably because of the changing character of infections. Such cases were common in the early twenties in Great Britain, but have become rare in recent years. A fall in complement titre in focal nephritis would be further evidence for considering the two as variations of a common disease.

The possibilities of the test in distinguishing an acute attack from a recurrence and in determining the pathogenesis of the progressive case have been referred to above.

Finally, the work of Thomson, Arnott and Mathew (1939) showing normal levels of complement in the toxaemias of pregnancy is valuable evidence against an aetiological relationship between this condition and acute nephritis.

SUMMARY.

Attention has been called to previous work indicating a fall in serum complement level in the early stages of acute nephritis.

A series of observations on 16 cases of acute nephritis is reported in confirmation.

Certain theoretical and practical applications of the test are indicated.

The co-operation of the honorary and resident staffs of the Royal Prince Alfred Hospital and the Royal Alexandra Hospital for Children in making their cases available for this study is gratefully acknowledged.

Thanks are due to Dr. Edgar Thomson, of the Fairfax Institute of Pathology, Royal Prince Alfred Hospital, for the supply of materials mentioned, and to Dr. Stephen Williams, of the Department of Pathology, Royal Alexandra Hospital for Children, for supply of many of the sera for the control series.

REFERENCES.

Coburn, A. F.—(1936) *Lancet*, ii, 1025.
Ecker, E. C., Seifter, S., and Duzois, T. F.—(1945) *J. Lab. clin. Med.*, **30**, 39.
Gunn, W. C.—(1914) *J. Path. Bact.*, **19**, 155.
Kellett, C. E.—(1936) *Lancet*, ii, 1262.
Idem and Thomson, J. G.—(1939) *J. Path. Bact.*, **48**, 519.
Payne, W. W., and Illingworth, R. S.—(1940) *Quart. J. Med.*, **9**, 37.
Pohl, A. W., and Rutstein, D. D.—(1944) *J. clin. Invest.*, **23**, 177.
Rachmilewitz, M., and Silberstein, W.—(1937) *J. Lab. clin. Med.*, **22**, 1240.
Rutstein, D. D., and Walker, W. H.—(1942) *J. clin. Invest.*, **21**, 347.
Thomson, S., Arnott, W. M., and Matthew, G. D.—(1939) *Lancet*, ii, 734.
Veil, W. H., and Buchholz, B.—(1932) *Klin. Wschr.*, **11**, 2019.

THE SYNTHESIS OF "FOLIC ACID" BY *STREPTOBACTERIUM PLANTARUM* AND ITS INHIBITION BY SULPHONAMIDES.

R. H. NIMMO-SMITH, J. LASCELLES* AND D. D. WOODS.

From the University Department of Biochemistry, Oxford.

Received for publication May 14, 1948.

THE hypothesis that the sulphonamides inhibit, in a competitive manner, the further utilization of *p*-aminobenzoic acid (Woods, 1940) is now generally accepted. The publication of the structure of synthetic liver *Lactobacillus casei* factor (pteroylglutamic acid) by Angier, Boothe, Hutchings, Mowat, Semb, Stokstad, SubbaRow, Waller, Cosulich, Fahrenbach, Hultquist, Kuh, Northey, Seeger, Sickels and Smith (1946) strongly suggested that *p*-aminobenzoate is involved in the metabolism of this and similar compounds, and that the sulphonamides might reasonably be expected to interfere with their biosynthesis. Evidence in support of this view was provided by Lampen and Jones (1946), who found that organisms which have an absolute requirement for factors which can be replaced by pteroylglutamic acid are highly resistant to sulphonamide inhibition when grown in the presence of amounts of this compound sufficient to promote growth in the absence of sulphonamides. Further, studies on certain enterococci whose growth was not dependent upon, but was stimulated by, added pteroylglutamic acid revealed the usual competitive reversal of sulphonamide inhibition by *p*-aminobenzoic acid and also a non-competitive reversal by pteroylglutamic acid. The same authors (Lampen and Jones, 1947) showed a similar effect with *Lactobacillus arabinosus* and *Streptobacterium plantarum*. Independently, Nimmo-Smith and Woods (1947) described non-competitive reversal of sulphonamide inhibition by pteroylglutamic acid with the latter organism, and also with *Clostridium acetobutylicum*. These results led to the conclusion that, at any rate for the above organisms, pteroylglutamic acid was the product of the reaction inhibited by sulphonamides or was sufficiently like it to be converted to the active compound (and that such conversion, if it occurred, was not inhibited by sulphonamides).

Evidence of the synthesis, during growth, of substances with the activity of pteroylglutamic acid has been found in all organisms tested which do not require preformed pteroylglutamic acid for growth (reviewed by Jukes and Stokstad, 1948). With a mixed culture from chick duodenum (Briggs, Luckey, Mills, Elvehjem and Hart, 1943) and with *Lb. arabinosus* (Sarett, 1947) the production of *Lb. casei* factor was stimulated by adding *p*-aminobenzoic acid to the culture medium. Finally, the synthesis of *Lb. casei* factor by growing cultures of parent and sulphonamide-resistant strains of *Bacterium coli* (Miller, 1944; Miller, Bruno and Berglund, 1947), and of a strain of yeast (Eppright and Williams, 1946), has been shown to be much reduced in the presence of sulphonamides.

Against this background it was of interest to study the bacterial synthesis

* Exhibition of 1851 Overseas Scholar.

of *Lb. casei* factor, if possible by non-growing suspensions, and to make a quantitative study of the effect of the sulphonamides on such synthesis. For this study we chose *Streptobacterium plantarum*, strain 5S, because it grows very poorly on a defined medium unless *p*-aminobenzoic acid is added, and because we already had quantitative data on the effect of sulphonamides on its growth. A preliminary report of the present work has already been made (Nimmo-Smith, Lascelles and Woods, 1948). It was first established that *Streptobacterium plantarum* could form much *Lb. casei* factor when suspended in a defined growth medium. Elimination from this medium of all constituents which were not found necessary or stimulatory for the synthesis led to the development of a greatly simplified suspending medium. Use of this technique allowed of a quantitative study of the synthesis of the factor by non-growing cells, and of a more detailed analysis of conditions affecting it, than would have been possible in the full growth medium.

METHODS.

Organisms.

Streptobacterium plantarum, strain 5S, kindly supplied by Dr. Niels Nielson of the Carlsberg Laboratories, was maintained as a stab culture in medium A, grown at 30°C. Subcultivations of the stock culture were made weekly, and fresh stab cultures made before each experiment.

Lactobacillus casei (*Lactobacillus helveticus*), American Type Culture Collection No. 7469, received *via* Dr. Barton-Wright, was maintained by weekly stab culture in Medium B 1, grown at 37° C.

Media.

Medium A.—Marmite, 5 g.; glucose, 2·5 g.; sodium acetate trihydrate, 5 g.; agar, 7·5 g.; water to 500 ml. pH adjusted to 6·7.

Medium B 1.—Tryptose (Difco), 5 g.; yeast extract (Difco), 1 g.; liver extract, 50 ml.; K_2HPO_4, 2·5 g.; $MgSO_4.7H_2O$, 100 mg.; $FeSO_4.7H_2O$, 5 mg.; $MnSO_4.4H_2O$, 5 mg.; $CaCO_3$, 1·5 g.; agar, 7·5 g.; water to 500 ml. The liver extract was prepared according to Nymon and Gortner (1946).

Medium B 2.—As medium B 1, with the omission of $CaCO_3$ and agar, the pH being adjusted to 6·7 to 6·9. This medium was found to contain about 25 mμg./ml. of *Lb. casei* factor (in terms of pteroylglutamic acid).

Medium C.—Casein hydrolyzate (Ashe Laboratories Ltd.; vitamin-free), 5 g.; DL-cysteine, 100 mg.; DL-tryptophan, 100 mg.; ammonium acetate, 5 g.; glucose, 20 g.; KH_2PO_4, 2 g.; $MgSO_4.7H_2O$, 400 mg.; ferric citrate, 35 mg.; $MnSO_4.4H_2O$, 10 mg.; aneurin hydrochloride, 100 μg.; pyridoxin hydrochloride, 1 mg.; biotin, 2 μg.; riboflavin, 300 μg.; water (glass-distilled) to 800 ml.; pH adjusted to 6·5. Quantities are for 1 l., the final adjustment to volume being made by the addition of sample (0·5 ml.) and inoculum (0·5 ml.) to each tube (4·0 ml.).

Streptobacterium plantarum grew very poorly in this medium unless *p*-aminobenzoic acid was added.

Medium D.—The assay medium for *Lb. casei* factor was based on that described by Roberts and Snell (1946), with the following modifications. The digestion of casein (Glaxo; vitamin-free, alcohol-extracted) was allowed to proceed

for only 24 hours, using 1 g. of a trypsin preparation (Difco) for each 100 g. casein. A fresh digest was prepared about every fortnight, and stored at 1°C. Synthetic DL-cysteine was substituted for L-cystine (at twice the concentration), uracil was omitted as it was without effect, and asparagine was added at the rate of 50 mg./l. final medium. All media were autoclaved for 10 minutes at 10 lb./sq. in.

There was some difficulty in preparing the casein digest and successive batches did not always give reproducible results. It was necessary to treat the digest adequately with charcoal to remove traces of *Lb. casei* factor : yet if the charcoal treatment was too extensive, unidentified factors were removed, and a poor assay medium resulted. Thus inadvertent changes in filtration time altered the duration of charcoal treatment, and upset this somewhat empirical balance. When, however, a rigid routine was followed, a very satisfactory assay medium could be produced.

Chemicals.

All chemicals were originally of the highest grade obtainable and, with the exception of those used in making media A and B, were further purified. In general, inorganic compounds and organic compounds of synthetic origin were re-crystallized once, whilst organic compounds of natural origin were recrystallized thrice. A.R. grade glucose was found to be sufficiently free from the factors involved in the present work, and was not recrystallized.

The components of medium C were shown by incorporation in media for *Acetobacter suboxydans* 621 or *Clostridium acetobutylicum*, or both, to contain negligible amounts of *p*-aminobenzoic acid. Tests with *Lb. casei* showed them also to be free from detectable amounts of *Lb. casei* factor.

Synthetic pteroylglutamic acid was received from the Lederle Laboratories Division of the American Cyanamid Company, Pearl River, New York, through the courtesy of Dr. Y. Subbarow. A stock solution of 100 μg./ml. in 0·01 N NaOH was stored at 0° C., diluted for each assay, and autoclaved in the assay medium after addition to the standard tubes.

Assay of Lb. casei *Factor.*

The conditions found by Stokes and Larsen (1945) to be optimal for extraction of *Lb. casei* factor from *Strept. faecalis* R were used in this investigation. No attempt was made, by enzymatic or other treatment, to assay other than the free form of the factor.

At the end of an experiment with *Streptobacterium plantarum* the suspension was centrifuged, the supernatant removed and the cells resuspended in a known volume (usually 5·0 ml.) of the assay medium (D). The cells were then killed by immersion in boiling water for 5–10 minutes. When it was required to assay the concentration of the factor in the suspending medium also, an aliquot of the supernatant was diluted with an equal volume of the assay medium. Finally, the tubes were autoclaved for 10 minutes at 10 lb./sq. in., cooled, centrifuged, and the clear supernatant used for assay. The *Lb. casei* factor in such extracts was stable for at least a fortnight in the refrigerator, as shown by repeated assays on the same extract.

The assays were conducted in 5 in. × 5/8 in. pyrex tubes, with a final volume

of 5·0 ml. medium D. The tubes were plugged with absorbent cotton-wool, since crude wool contains *Lb. casei* factor (Sherwood and Singer, 1944). The medium (4·0 ml. to be diluted to 5·0 ml.) was autoclaved for 10 minutes at 10 lb./sq. in. After cooling 0·5 ml. of suitably diluted sample was added. Several dilutions (usually three) of each sample, with duplicate tubes at each level, were assayed as a routine. Within each assay was included a standard set of tubes containing 4–6 levels of pteroylglutamic acid, with 4 tubes at each level, covering the range 0 to 0·8 mμg./5 ml.

The inoculum used was 0·5 ml. of a 1/100 dilution in water of a 24–26 hour culture of *Lb. casei* in the basal medium supplemented with 6 mμg./ml. pteroylglutamic acid. This culture had been inoculated with 2 loopfuls of a 16 hour culture in medium B 2, inoculated in turn from a fresh 24 hour stab culture in medium B 1. All incubations were at 37° C.

Preliminary experiments showed that a smaller blank and a sharper response curve were obtained when the tubes were incubated either in an atmosphere of 5 per cent CO_2 in H_2 or in 100 per cent CO_2, than when incubated in air, H_2 or N_2. Lowry and Bessey (1944) found a similar effect in riboflavin assays with this organism. In earlier experiments, assay tubes were incubated for 40 hours; later it was found possible to reduce this to 22 hours with no loss in precision, and probably a gain in accuracy (see Results). With a few exceptions, all results reported were obtained with the 22 hour assay.

Growth was measured turbidimetrically with the Hilger "Spekker" photoelectric absorptiometer, and a standard dose-response curve plotted. The amount of factor (in terms of pteroylglutamic acid) at each level of a sample was found by reading directly off the curve and taking the mean of duplicates. When, as was usual, more than one level fell on the useful part of the curve, the mean of the values at different levels was taken as the final figure.

The precision of the assay method in the useful part of the response curve has been computed for 14 consecutive assays. At the 0·2 mμg. level, the standard error of the mean of duplicate tubes was $\pm$ 0·012 mμg., and at the 0·4 mμg. level, $\pm$ 0·025 mμg. This corresponds with a coefficient of variation of about 6 per cent at each level. The standard error of the mean of unknowns was found not to differ significantly from that of the standard.

Preparation and Use of Suspensions of Streptobacterium plantarum.

Opacity.—The opacity of suspensions and cultures was measured with the photoelectric absorptiometer, and a calibration of dry weight against drum reading made.

Inoculum.—The inoculum was prepared by transferring from a 24 hour stab culture of *Streptobacterium plantarum* in medium B 1 into medium B 2, and incubating for 18 hours; a 1/25 dilution of this was either used directly as inoculum, or after a further 24 hours' subculture in medium C supplemented with 10^{-7} M *p*-aminobenzoic acid. Organisms obtained with either of these inocula appeared to be identical, so that the latter type was used in most of the experiments, as it reduced the risk of transferring *Lb. casei* factor from medium B 2.

Suspensions.—The medium (B 2 or C *plus* 10^{-7} M *p*-aminobenzoic acid) was tubed in 25 ml. quantities in 8 in. × 1 in. pyrex tubes, 0·25 ml. inoculum added, and incubated at 30° C. in an air-incubator for 17–18 hours, unless cultures of

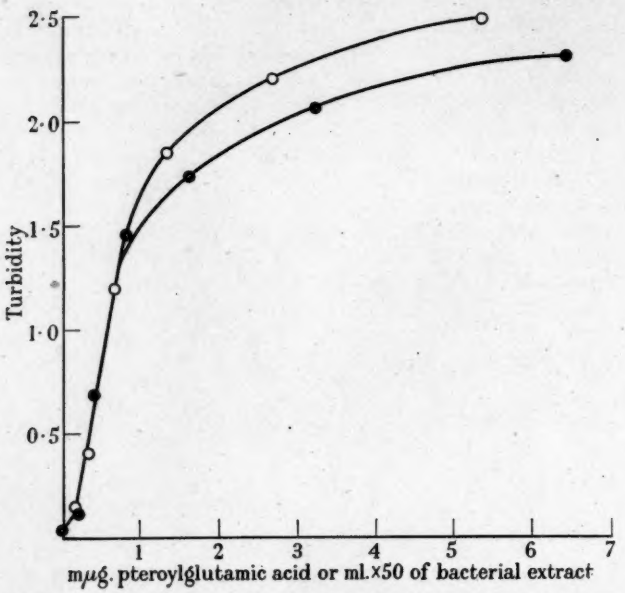

FIG. 1.—Response of *Lb. casei* to synthetic pteroylglutamic acid (full circles) and to *Sbm. plantarum* extract (open circles) after 40 hours' incubation. Each point represents the mean of quadruplicate tubes. Divergence of response at high doses is shown. (Turbidity = actual absorptiometer reading multiplied by dilution factor.)

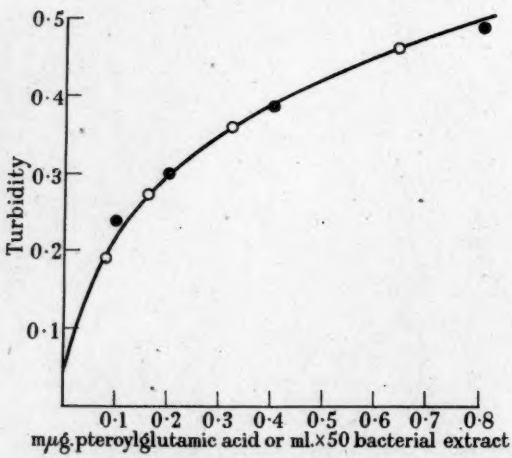

FIG. 2.—Response of *Lb. casei* to synthetic pteroylglutamic acid (full circles) and to *Sbm. plantarum* extract (open circles) after 20 hours' incubation. Each point represents the mean of quadruplicate tubes. (Turbidity = actual absorptiometer reading × dilution factor.)

a different age were required. The cells were centrifuged out, washed with the original culture-volume of 0·85 per cent NaCl and finally resuspended in the desired reaction medium. The opacity was measured at this stage (0·2 to 2·0 mg./ml. according to the experiment). The suspensions were then incubated in a water-bath at 30° C. If the cells were to be used for a second incubation, they were spun out and either resuspended in the fresh medium, and used at once for the second part of the experiment, or else stored overnight at 4° C. as a paste, or as a suspension in a small volume of medium C, lacking glucose, and with no *p*-aminobenzoic acid added. In the latter case the cells were spun out and washed with saline before use. Suspensions were usually incubated in 15 ml. centrifuge tubes at a final volume of 5·0 ml.; for larger volumes 1 in. test-tubes were used.

RESULTS.

Nature of the Factor.

The exact nature of the factor synthesized by *Streptobacterium plantarum* has not yet been investigated, nor has its identity with any of the known members of this group of growth factors been established chemically.

The response of *Lb. casei* to the bacterial extract and to synthetic pteroylglutamic acid was identical, within the limits of experimental error, until high dose-levels were reached (Fig. 1). Deviation at high levels did not necessarily indicate non-identity of the two compounds involved; nor did it rob the assay of validity, since over the "useful" part of the curve (Fig. 2) there was little or no discrepancy. The exaggerated response of *Lb. casei* to large doses of bacterial factor became greater as the assay time was increased, and at 40 hours was very marked. Possible reasons for this deviation are considered in the Discussion. Thus, by using only the steep part of the standard curve for incubation at 22 hours, a satisfactory estimate of the cell content of the factor was obtained. It should be emphasized that our main concern was for the relative content under various conditions, and that no claim is made for absolute amounts; expression in terms of mμg. pteroylglutamic acid/mg. dry weight of cell has been used merely as a convenience.

Concentration of the Factor in Freshly Harvested Cells.

Assays on extracts of cells freshly harvested from medium C *plus* *p*-aminobenzoic acid showed great variation from one culture to another; figures from thirteen consecutive experiments have been collected in Table I. The observed variation could not be correlated with age of culture or with the two concentrations of *p*-aminobenzoic acid used (10^{-6} and 10^{-7} M). On the other hand, although the number of experiments is not yet large enough for an accurate evaluation, the content of the factor in cells grown on medium B 2 seemed to be distinctly lower.

Conditions Necessary for Optimal Synthesis of the Factor.

Preliminary Incubation.

When cells, freshly harvested and washed, were incubated in the full growth medium C, containing *p*-aminobenzoic acid, little or no increase in content of the *Lb. casei* factor was found. This failure was thought possibly to be due to

TABLE I.—*Disappearance of Preformed* Lactobacillus casei *Factor.* Washed cells of *Sbm. plantarum* incubated in growth medium C in the absence of *p*-aminobenzoic acid.

Exp.	Incubation time (hr.).	*Lb. casei* factor (mμg./mg.)	
		Initial.	After incubation.
1	6	10·4	0·72
2	6	6·4	0·12
3	3	5·0	0·50
4	2	1·7	0·24
5	2	12·0	0·75
6	2	29·0	1·70
7	2	5·7	4·0
8	2	14·0	9·1
9	6	6·6	0·30
10	4	14·0	1·4
11	3	7·9	0·80
12	2	12·0	3·0
13	3	4·9	2·0
Mean	.	9·97	1·89
Standard error of mean		1·91	0·68

an already high content preventing further synthesis. Accordingly, attempts were made to obtain cells with a lower initial content. Incubation in medium C, without *p*-aminobenzoic acid, was successful in this respect, for the initial cell content fell greatly, usually by more than 90 per cent, during incubation for 2 to 6 hours (Table I).

Strikingly, when such cells were further incubated in a medium containing *p*-aminobenzoic acid there was considerable synthesis of the factor (up to 50 mμg./mg.). Such increase, to many times the content of freshly harvested cells, suggested that the initial inability to synthesize it was not due to "saturation."

TABLE II.—*Effect of Glucose on the Removal of the Factor.*

Washed cells of *Sbm. plantarum* suspended in medium C (*minus* glucose) and incubated with and without added glucose.

Exp.	Incubation time (hr.).	*Lb. casei* factor (mμg./mg.).		
		Initial.	After incubation in	
			No glucose.	0·02 M glucose.
1	3·5	14·0	6·9	1·4
2	0·75	6·6	6·3	1·1
	1·5	6·6	4·0	0·8
3	2·0	14·0	9·1	1·3
4	2·0	29·0	26·0	1·7

Disappearance of preformed factor from the cells was not due to escape into the suspending medium. For instance, of 60 mμg. disappearing from 7·5 mg. cells, only 0·5 mμg. could be found in the reaction medium.

Removal of the factor depended on concurrent glycolysis and there was little loss in medium C without glucose (Table II). In short-term experiments, disappearance of factor increased with increasing glucose concentration, and was maximum at 0·02 M glucose (Fig. 3).

Since incubation in *p*-aminobenzoic acid-free medium resulted not only in an almost complete disappearance of the preformed factor, but also in an apparently enhanced ability to synthesize more, this procedure was used throughout the following work unless otherwise stated. Cells so treated maintained full ability to synthesize the factor when stored for up to 24 hours at 4° C. either as a paste, or as a heavy suspension in medium C.

It is clear that cells of *Streptobacterium plantarum* synthesize *Lb. casei* factor when incubated in a growth medium (C) containing *p*-aminobenzoic acid. The

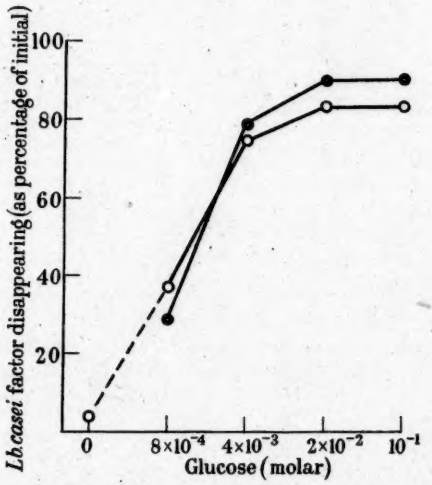

FIG. 3.—Effect of glucose concentration on disappearance of preformed factor. Cells incubated in medium C (*minus* glucose) for 3 hours (Exp. 1, open circles) and for 6 hours (Exp. 2, full circles).

question as to which constituents of this medium are essential for the synthesis was next investigated. Such information might throw light on the mechanism, and also show whether the process was dependent on cell division. On the growth medium, even thick suspensions grew to some extent, though the opacity was never more than doubled.

Simplification of Reaction Medium.

Omission from medium C of casein hydrolyzate (provided glutamic acid was added back), and the inorganic salts of Fe***, Mn** and Mg** did not decrease the amount of factor synthesized under standard conditions; it was concluded that they were either non-essential or required in such low concentrations that the amounts already present in the cells were adequate. Withdrawal of the

growth factors was without effect, and similar conclusions were drawn with regard to them.

As a result of these observations a simplified medium, in which the organism would synthesize even more factor than in medium C (Table III), was used for further experiments. Basically the simplified medium contained buffer (usually 0·04 M phosphate, pH 6·5); glucose, 0·1 M; DL-glutamate, 0·01 M; *p*-aminobenzoate, 10^{-4} M.

TABLE III.—*Substances Involved in the Synthesis of the Factor.*

The simplified systems also contained ammonium acetate and KH_2PO_4 in the concentrations present in medium C.

	Reaction medium.				
Medium C	+	−	−	−	−
DL-Glutamate 0·01 M	−	+	+	−	+
Glucose 0·1 M	+	+	−	+	+
p-Aminobenzoate 10^{-4} M	+	−	+	+	+

Exp.	Incubation (hr.).	*Lb. casei* factor synthesized (mμg./mg.).				
1	3.	5·7	1·4	1·5	2·0	19
2	3.	6·5	1·0	3·4	8·1	20
	6.	4·9	0·9	0·7	7·8	33

p-*Aminobenzoic acid.*—In the absence of added *p*-aminobenzoate no factor was synthesized (Table III); as would be expected, the decrease in preformed factor found during preliminary incubation tended to continue. Between a concentration of 10^{-8} and 10^{-7} M an increase in the factor could be detected in short experiments (Table IV); this corresponds very closely with previous

TABLE IV.—*Effect of* p-*Aminobenzoic Acid Concentration on the Synthesis of the Factor.*

Washed cells of *Sbm. plantarum* suspended in reaction medium containing 0·04 M phosphate buffer, 0·1 M glucose and 0·01 M DL-glutamate.
Time of incubation, 4 hours.

p-aminobenzoic acid (M)	*Lb. casei* factor (mμg./mg.). 10^{-8}	10^{-7}	10^{-6}
Exp. 1	1·1	17	16
,, 2	2·5	25	55
,, 3	—	18	82

— = not tested.

findings in growth experiments with this organism (Nimmo-Smith and Woods, 1947) where 10^{-7} M *p*-aminobenzoate appeared to be just optimal and 10^{-8} M definitely sub-optimal. As the concentration was increased logarithmically from 10^{-7} M to 10^{-4} M there was a progressive increase in the amount of the factor synthesized (Fig. 4). Increasing the *p*-aminobenzoate concentration up

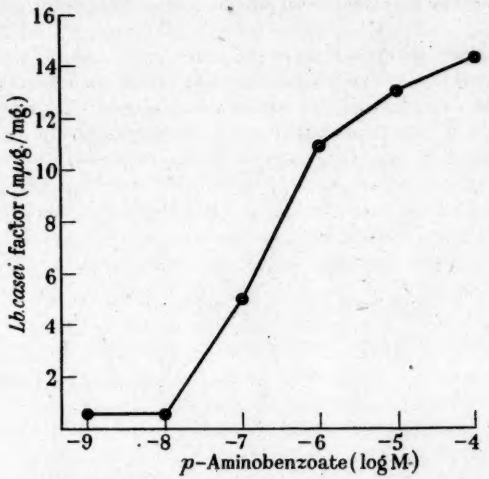

FIG. 4.—Effect of *p*-aminobenzoate concentration on the synthesis of factor. Cells suspended in 0·04 M phosphate buffer, pH 6·5, 0·1 M glucose and 0·01 M DL-glutamate for 4 hours.

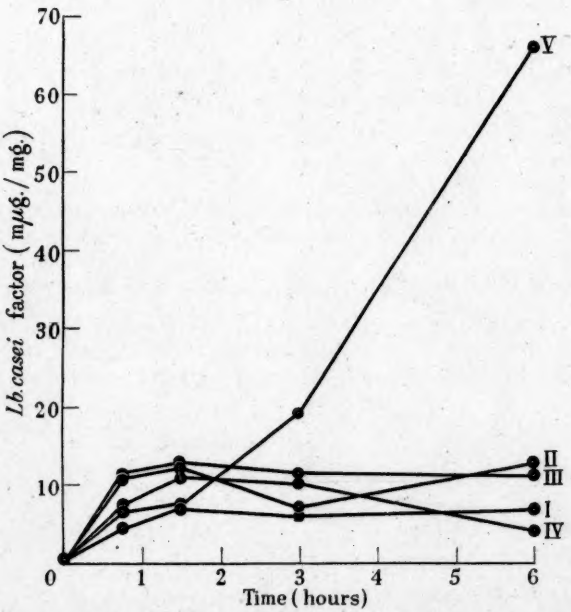

FIG. 5.—Effect of glucose concentration on the synthesis of the factor. Cells suspended in medium C (*minus* glucose) containing 10^{-4} M *p*-aminobenzoate and incubated with: I, no glucose; II, 8×10^{-4} M; III, 4×10^{-3} M; IV, 2×10^{-2} M; and V, 10^{-1} M glucose.

to 10^{-2} M produced no further increase; nor was there any inhibition by these high concentrations.

Glucose.—This was essential for maximum synthesis (Table III). Without added glucose a small increase, followed by a decrease in the concentration of the factor, was sometimes found. There was no clear-cut relationship between glucose concentration and the amount of the factor formed. In general, synthesis soon ceased when the glucose concentration was low (e.g. 0·001 M) or zero, and tended to continue for a longer time with increasing concentration. Simultaneous measurements of the pH of the suspending medium suggested that synthesis ceased at about the same time as active glycolysis. A further increase from 0·02 M to 0·1 M glucose gave a marked increase both in the total amount of the factor formed, and in the duration of the synthesis (Fig. 5; Table V).

TABLE V.—*Effect of Glucose on the Synthesis of the Factor.*

Exp. 1–3: Washed cells of *Sbm. plantarum* suspended in 0·04 M phosphate buffer, pH 6·5, 0·01 M DL-glutamate and 10^{-4} M *p*-aminobenzoic acid.

Exp. 4–6: Cells suspended in medium C (*minus* glucose) with 10^{-4} M *p*-aminobenzoic acid.

Exp.	Incubation time (hr.).	*Lb. casei* factor formed (mμg./mg.). No glucose.	0·1 M glucose.
1	3	5·6	9·7
2	3	3·4	20
	6	0·7	33
3	3	1·5	19
4	3	6·6	66
5	3	3·0	12
6	3	6·5	15

Medium C contained *ca.* 0·1 M glucose.

Glutamic acid.—On most occasions the addition of DL-glutamate produced a twofold to fivefold increase in the amount of the factor synthesized (Table VI).

TABLE VI.—*Effect of Glutamate on Synthesis of the Factor.*

Summary of 17 paired experiments, duration 3–8 hours. Cells of *Sbm. plantarum* suspended in phosphate, acetate or phosphate *plus* acetate buffer, pH 5·0–6·5; DL-glutamate 0·02 M or 0·004 M; *p*-aminobenzoate 10^{-4} M; glucose 0·1 M.

	Lb. casei factor (mμg./mg.). Glutamate. Added.	Not added.	Difference.
Mean	15·5	7·3	8·2
Standard error of mean	2·5	1·2	3·0

Although conditions between experiments were varied considerably the overall comparison was made legitimate by pairing within each experiment.

Sometimes, however, no increase was observed after adding glutamate up to 0·02 M. In those experiments where stimulation by glutamate was found, there was not always a correlation between the amount of factor synthesized, and the concentration of glutamate added (8×10^{-4} M to 2×10^{-2} M), though this was sometimes the case. Nevertheless, an analysis of the combined results of all the experiments (seventeen) carried out (Table VI) shows clearly that glutamate certainly stimulates the synthesis of *Lb. casei* factor under the present conditions, and may be an essential component of the system.

The Simplified Medium.

As a result of all these observations, the simplified medium, as stated above, was developed. It seems probable that the synthesis of the *Lb. casei* factor by *Streptobacterium plantarum* can take place in the absence of cell division, for turbidimetric readings showed that no change in opacity occurred when cells were incubated in the simple medium, even for as long as 24 hours.

As stated earlier, large increases in the cell content of the factor have only been found with cells receiving a preliminary incubation in medium C. When this first incubation was carried out in buffered solutions containing only glucose and DL-glutamate the cells failed almost completely to synthesize further factor when *p*-aminobenzoic acid was added subsequently. Indeed this was also the case when medium C *plus* *p*-aminobenzoate was used for the second incubation (Table VII). Moreover, the removal of preformed factor appeared to be less

TABLE VII.—*Effect of Preliminary Incubation in Different Media on Subsequent Synthesis of Factor.*

Simple medium : 0·04 M phosphate buffer pH 6·5 (Exp. 1) or 0·04 M acetate buffer, pH 5·0 (Exp. 2); glucose 0·1 M, DL-glutamate 0·01 M.
C = medium C.
For first incubation (4 hr.) neither medium contained *p*-aminobenzoate; for second incubation (4 hr.) both contained 10^{-4} M.

	First incubation.			Second incubation.	
Exp.	Medium	Factor (mμg./mg.). Initial.	Final.	Medium.	Factor increase. (mμg./mg.).
1	C	3·3	0·63	Simple	10·8
	Simple	3·6	2·0	,,	0·3
2	C	2·6	1·5	C	7·2
				Simple	15·3
	Simple	7·8	5·6	C	0·3
				Simple	0·7
	No first incubation			Simple	0·7

efficient in the simple medium (Table VII). The constituents of medium C involved in maintaining cells capable of synthesizing the factor have not yet been identified.

The Influence of pH.

The investigation of the effect of altering pH presented considerable difficulties, as *Streptobacterium plantarum* ferments glucose at a very rapid rate, and even in the presence of 0·1 M buffer solution, the pH was rapidly lowered by the organism in the presence of glucose. Glucose could not be omitted because of its necessity for the synthesis of the factor. In spite of these difficulties, it was possible to conclude that synthesis was greater at relatively low pH values (pH 4 to 5). Further, experiments in 0·04 M acetate buffer showed that a significantly greater amount of the factor was synthesized at an initial pH of 4·6 than at pH 3·8 or pH 5·6 (Table VIII).

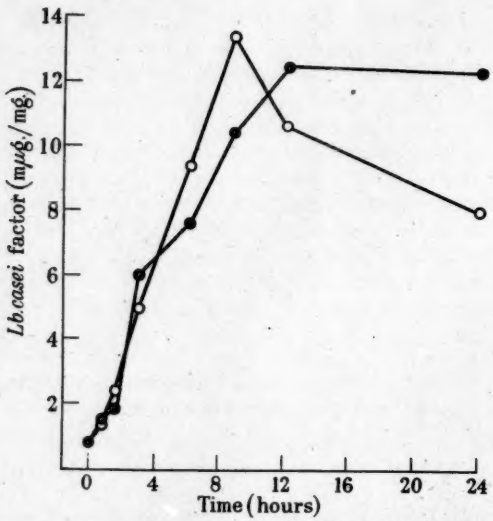

FIG. 6.—Two experiments showing the course of the synthesis in the simplified reaction medium.

TABLE VIII.—*Effect of pH on the Synthesis of the Factor.*

Incubation was for 4 hours in 0·04 M acetate buffer containing glucose 0·1 M. DL-glutamate, 0·01 M, *p*-aminobenzoate 10^{-4} M and phosphate 0·004 M.

Exp.	Factor (mμg./mg.) formed at pH.		
	3·8	4·6	5·6
1	12	15	9·8
	15	17	6·8
2	11	37	10
	14	21	14

pH values are those obtaining initially.

Course of the Reaction.

In the simple reaction medium increase in the cell content of the factor was almost linear for about 12 hours (Fig. 6). After this the concentration remained

steady or began to decline. No observations were made beyond 24 hours. No true "lag" period was observed, although in some experiments the maximal rate of synthesis did not seem to be reached until after about 4 hours' incubation.

In short experiments the amount of factor synthesized was proportional, within the limits of experimental error, to the amount of bacteria added, i.e.

TABLE IX.—*Relationship between Quantity of Bacteria used and Amount of Factor Synthesized.*

Cells of *Sbm. plantarum* suspended in 0·04 M phosphate buffer, pH 6·5, glucose 0·1 M, DL-glutamate 0·01 M and *p*-aminobenzoate 10^{-4} M.

Dry wt. of organism (mg./5·0 ml.).	*Lb. casei* factor (mμg./mg. organism) formed after (hr.).	
	2·5	5·0
11·2	4·6	6·4
5·6	4·2	5·7
2·8	3·8	4·6
1·4	4·0	3·9
0·7	3·4	11

the yield per unit cell mass was constant (Table IX). In longer experiments some irregularities were observed with the more dilute suspensions.

Influence of Type of Culture Medium and Cell Age.

Cells grown in the complex medium B 2, treated in the same way as, and compared directly with, cells from the defined medium C were found also to be able to synthesize the factor, but at about only one half the rate with the latter. Because the organism grew so much faster in the complex medium, as well as for more fundamental reasons, cells of different ages were compared for their ability to synthesize *Lb. casei* factor. On three occasions cells harvested from cultures in medium C at 48 hours, 24 hours (both fully grown), 18 hours (towards the end of the logarithmic phase of growth), and 12 hours (in the logarithmic phase) were compared. These experiments, however, revealed no consistent difference in synthetic ability in relation to the physiological age of the cell.

Increasing the concentration of *p*-aminobenzoic acid in medium C during growth from 10^{-7} M to 10^{-6} M appeared to be without effect both on the amount of factor present in cells at the end of growth, and on the amount which they were subsequently able to synthesize.

Inhibition by Sulphonamides.

Both sulphanilamide and sulphathiazole completely inhibited synthesis of the factor under the standard conditions. Other sulphonamides were not tested. The inhibition was completely overcome by increasing the concentration of *p*-aminobenzoic acid. In experiments in which both sulphonamides were used over a 10,000-fold range of concentration it was found that the reversal by *p*-aminobenzoic acid was of a strictly competitive nature. The results of a typical experiment with sulphathiazole are summarized in Fig. 7.

Previous exposure to sulphonamide did not diminish the ability of the organism subsequently to synthesize the factor. Cells incubated for 6 hours in the presence of either 10^{-3} M sulphanilamide or 10^{-4} M sulphathiazole, and in the absence of *p*-aminobenzoate were centrifuged, washed with saline, and re-suspended in a simple medium containing *p*-aminobenzoate. Just as much factor was synthesized as with cells which had been smilarly pre-incubated in the absence of

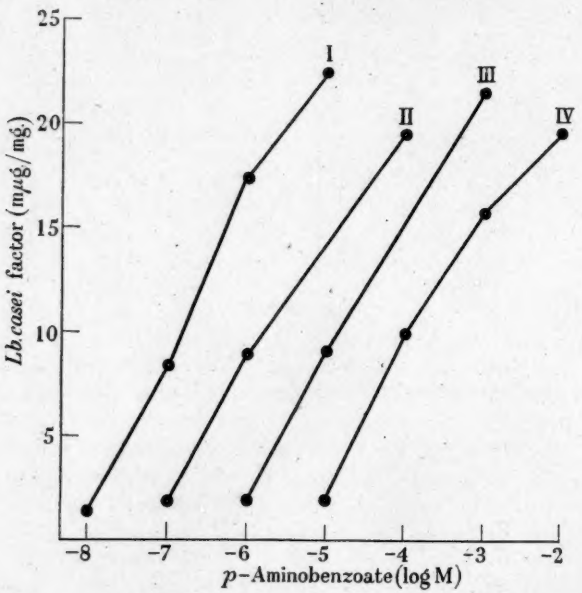

FIG. 7.—Inhibition of synthesis of the factor by sulphathiazole and its reversal by *p*-aminobenzoic acid. Cells incubated in simplified medium for 4 hours. Sulphathiazole conc.: I, 10^{-7} M; II, 10^{-6} M; III, 10^{-5} M; IV, 10^{-4} M.

TABLE X.—*Effect on Synthesis of the Factor of Preliminary Incubation in the Presence of Sulphanilamide.*

Conditions: First incubation in medium C; second incubation in simplified reaction medium.

Lb. casei factor (mμg./mg.)

	Sulphanilamide.			
	0.		10^{-3} M.	
	Initial.	Final.	Initial.	Final.
1st incubation (3 hr.)	11	2·2	11	4·5
	S-amide.		S-amide.	
	0.	10^{-3} M.	0.	10^{-3} M.
2nd incubation (4 hr.)	11	0·7	14	0·7

sulphonamide (Table X). This procedure did not alter the sensitivity of the synthetic mechanism to sulphonamide inhibition.

The sulphonamides did not significantly inhibit the removal of preformed factor which took place during incubation in the absence of *p*-aminobenzoate (Table X).

DISCUSSION.

There is evidence that the various members of the "folic acid" group of growth factors, although they have much in common, are not identical with each other. Thus Stokes and Larsen (1945) noted that the *Lb. casei* factor formed from *Strep. faecalis* R factor (rhizopterin) by *Strept. faecalis* R was much less stable than pteroylglutamic acid. Hall (1947), testing the activity of various preparations on acid production by *Strept. faecalis* R, concluded that they varied in detail in the type of response shown according to whether they were of animal or plant origin, and that pteroylglutamic acid corresponded most closely to the animal form. There appears to be no information on comparative responses to unconjugated "folic acid" of bacterial and other origin.

The response of *Lb. casei* to the factor as formed by *Streptobacterium plantarum* is remarkably similar, in the lower dose-range, to its response to pteroylglutamic acid ; it is only at higher levels that significant differences are observed (Fig. 1). Bird and Robbins (1946) noted a similar phenomenon during assays for vitamin B_c in crude preparations, although crystalline vitamin B_c gave a response indistinguishable from that of pteroylglutamic acid. These workers produced strong evidence that such abnormal response-curves were due to crude samples (such as those with which we were concerned) containing a mixture of free and conjugated vitamin (Pfiffner, Calkins, O'Dell, Bloom, Brown, Campbell and Bird, 1945), for (*a*) a sample of B_c conjugate (pteroylheptaglutamic acid) gave a response which was exaggerated in the manner of that given by the crude samples, and (*b*) such crude samples, after treatment with an enzyme preparation which hydrolyzed conjugates, gave the same response with *Lb. casei* as did pteroylglutamic acid. The differences in response to pteroylglutamic acid and to crude preparations could be explained on the basis of the conjugate content of the latter and by assuming the ability of *Lb. casei* to split the conjugate. This explanation could possibly be extended to our own observations, where the divergence of response curves at high doses could be due to the increasing effect of a conjugate, superimposed on the response to a factor with properties very similar (as regards *Lb. casei*) to pteroylglutamic acid. At least one conjugated form of the factor of bacterial origin is known, the "fermentation *Lb. casei* factor" (pteroyltriglutamic acid), (Hutchings, Stokstad, Bohonos and Slobodkin, 1944).

Conjugate formation by *Streptobacterium plantarum* may account for disappearance of preformed factor during incubation in the absence of *p*-aminobenzoic acid. The requirement for a simultaneous breakdown of glucose would fit in with the view that the removal is an anabolic process. It seems likely that in the freshly harvested cell at least two phases of the metabolism of the factor are manifest, one in which the factor is synthesized in a form active for *Lb. casei* and for which a supply of energy, *p*-aminobenzoic acid and possibly glutamate is required, and a second, for which energy, and possibly glutamate, is needed, in which the factor is converted to a form inactive or only partially active for the assay organism. *Lb. casei* responds fairly well to pteroyltriglutamic

acid (Hutchings *et al*, 1944), but not at all to pteroylheptaglutamic acid (Pfiffner *et al*, 1945). If the two processes proceeded at the same rate, then a dynamic equilibrium would be set up and the concentration of the factor as assayed by *Lb. casei* would appear to remain constant. Just such a situation might exist in freshly harvested *Streptobacterium plantarum*, and explain the failure to obtain an increase in the factor when such cells are incubated in the presence of *p*-aminobenzoic acid ; omission of the *p*-aminobenzoate (since the organism can synthesize it slowly if at all) would allow the second phase to proceed in the absence of the first, resulting in a disappearance of the factor. Since, on this hypothesis, *p*-aminobenzoate is not directly involved in the removal of the factor, the sulphonamides would not be expected to interfere with the second phase, and this is confirmed by experimental observation.

Even on this hypothesis, however, it is not yet possible to explain the ability of cells receiving a preliminary incubation in the absence of *p*-aminobenzoic acid to synthesize factor on subsequent incubation in its presence. It is unlikely that it is due to liberation of active factor from a combined form since (*a*) no increase is observed in the absence of *p*-aminobenzoate, (*b*) the increase is inhibited by sulphanilamide and sulphathiazole, and (*c*) simultaneous glycolysis is necessary ; there has been no suggestion that liberation of free *Lb. casei* factor from its conjugates requires a source of energy. One possible explanation is that during the preliminary incubation, the enzyme system responsible for the hypothetical second phase of metabolism of the factor undergoes damage, either due to direct injury of an enzyme or through loss of a co-factor, thus preventing or slowing down removal of free factor formed in the first phase. Further work is being undertaken to test this hypothesis, and to throw more light on the problem of the disappearance of the factor.

Although it was established, over a long series of experiments, that addition of glutamate significantly increased the amount of factor formed, this effect was rather unpredictable. Gale (1947) and Taylor (1947) have shown that Gram-positive bacteria are capable of accumulating glutamic acid within the cell. It therefore seems likely that in preliminary incubation in medium C *Streptobacterium plantarum* may assimilate variable amounts of glutamate, and sometimes carry over to the second incubation enough stored glutamate to provide for the requirements of synthesis of *Lb. casei* factor ; on such occasions added glutamate would not be expected to stimulate the synthesis. Furthermore, although there are as yet no relevant experimental data, it is to be expected that the synthesis of conjugates of the factor requires glutamic acid. Thus, if conjugate formation is indeed occurring in *Streptobacterium plantarum*, and this to some extent persists during the second incubation (in the presence of *p*-aminobenzoic acid), addition of glutamate might be expected to stimulate this phase of the metabolism of the factor, as well as the first phase (formation of the free factor). Under these conditions addition of glutamate would not stimulate an increased concentration of the free factor unless the second metabolic phase was severely suppressed (e.g. by fairly prolonged preliminary incubation).

For the synthesis of the pteridin moiety of the molecule, no specific addition appears to be necessary ; since, however, this part of the synthesis has not been studied it would be fruitless to speculate at this point.

In this investigation we have attempted to clarify the position with regard to the biosynthesis of a group of growth factors essential not only for bacteria,

but also for higher animals, including probably man (reviewed by Jukes and Stokstad, 1948), and have provided direct evidence to support the view (Lampen and Jones, 1947) that the inhibition of the biosynthesis of this factor is alone sufficient to account for the inhibition of growth of certain organisms by the sulphonamides.

SUMMARY.

1. The synthesis of a *Lactobacillus casei* factor ("folic acid") by washed suspensions of *Streptobacterium plantarum* has been studied.

2. For the synthesis, only glucose and *p*-aminobenzoic acid have been found essential; glutamic acid is stimulatory.

3. An increase in the cell content of the factor is not found unless the cells are first incubated in a *p*-aminobenzoic acid-free medium.

4 Synthesis of the factor is inhibited by sulphonamides; this inhibition is overcome in a strictly competitive manner by *p*-aminobenzoic acid

We wish to express our gratitude to the Medical Research Council for a full-time grant to one of us (R. H. N-.S.) during part of the work.

We have much pleasure in giving thanks also to Miss K. Sims and to Miss M. Pollicott for technical assistance.

REFERENCES.

ANGIER, R. B., BOOTHE, J. H., HUTCHINGS, B. L., MOWAT, J. H., SEMB, J., STOKSTAD, E. L. R., SUBBAROW, Y., WALLER, C. W., COSULICH, D. B., FAHRENBACH, M. J., HULTQUIST, M. E., KUH, E., NORTHEY, E. H., SEEGER, D. R., SICKELS, J. P., AND SMITH, J. M.—(1946) *Science*, **103**, 667.

BIRD, O. D., AND ROBBINS, M.—(1946) *J. biol. Chem.*, **163**, 661.

BRIGGS, G. M., LUCKEY, T. D., MILLS, R. C., ELVEHJEM, C. A., AND HART, E. B.—(1943) *Proc. Soc. exp. Biol., N.Y.*, **52**, 7.

EPPRIGHT, M. A., AND WILLIAMS, R. J.—(1946) *J. gen. Physiol.*, **30**, 61.

GALE, E. F.—(1947) *J. gen. Microbiol.*, **1**, 53, 327.

HALL, D. A.—(1947) *Biochem. J.*, **41**, 287, 294.

HUTCHINGS, B. L., STOKSTAD, E. L. R., BOHONOS, L., AND SLOBODKIN, N. H.—(1944) *Science*, **99**, 370.

JUKES, T. H., AND STOKSTAD, E. L. R.—(1948) *Physiol. Rev.*, **28**, 51.

LAMPEN, J. O., AND JONES, M. J.—(1946) *J. biol. Chem.*, **166**, 435.—(1947) *Ibid.*, **170**, 133.

LOWRY, O. H., AND BESSEY, O. A.—(1944) *Ibid.*, **155**, 71.

MILLER, A. K.—(1944) *Proc. Soc. exp. Biol., N.Y.*, **57**, 151.

Idem, BRUNO, P., AND BERGLUND, R. M.—(1947) *J. Bact.*, **54**, 9.

NIMMO-SMITH, R. H., LASCELLES, J., AND WOODS, D. D.—(1948) *J. gen. Microbiol.*, (in the press).

Idem AND WOODS, D. D.—(1947) *Absts., XVII Int. physiol. Congr.*, 328.

NYMON, M. C., AND GORTNER, W. A.—(1946) *J. biol. Chem.*, **163**, 277.

PFIFFNER, J. J., CALKINS, D. G., O'DELL, B. L., BLOOM, E. S., BROWN, R. A., CAMPBELL, C. J., AND BIRD, O. D.—(1945) *Science*, **102**, 228.

ROBERTS, E. C., AND SNELL, E. E.—(1946) *J. biol. Chem.*, **163**, 499.

SARETT, H. P.—(1947) *Ibid.*, **171**, 265.

SHERWOOD, M. B., AND SINGER, E. D.—(1944) *Ibid.*, **155**, 361.

STOKES, J. L., AND LARSEN, A.—(1945) *J. Bact.*, **49**, 495.

TAYLOR, E. S.—(1947) *J. gen. Microbiol.*, **1**, 86.

WOODS, D. D.—(1940) *Brit. J. exp. Path.*, **21**, 74.